Convergence Of Proliferative And Survival Signals On The pRB/E2F Pathway In Haematopoietic Cells

Thesis

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Convergence of proliferative and survival signals on the pRB/E2F pathway in haematopoietic cells

Thesis submitted by

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Abstract

Chronic myeloid leukemia (CML) is a malignant stem cell disease characterised by an expansion of myeloid progenitor cells. These cells express a BCR/ABL fusion protein with constitutively activated tyrosine kinase activity, which causes a deregulation of apoptosis and cell cycle progression. In the pro-B BaF3 cell line, BCR/ABL has been shown to abrogate the IL-3 dependence to proliferate, but the signalling pathways activated by BCR/ABL and IL-3 to promote proliferation and survival are not yet well defined. In this study, BaF3 cells and a BaF3 cell line stably over-expressing BCR/ABL, BaF3-p210, were used to identify the downstream targets of BCR/ABL and IL-3. Both BCR/ABL and IL-3 were shown to induce the expression of cyclin D2 and inhibit the expression of the cell cycle inhibitor, $p27^{Kip1}$. This regulation was shown to be directly due to BCR/ABL, in haematopoietic cells, by two different approaches. First, using a BaF3 cell line (TonB210.1) where the BCR/ABL expression is inducible by doxycycline and second, by inhibiting the kinase activity of BCR/ABL with the Abl tyrosine kinase inhibitor STI571. In order to establish the functional significance of cyclin D2 and $p27^{Kip1}$ expression in response to IL-3 and BCR/ABL expression, the effects of ectopic expression of cyclin D2 and $p27^{Kip1}$ on cell proliferation and survival were studied. The results demonstrate that both cyclin D2 and $p27^{Kip1}$ have a role in BaF3 cell proliferation and survival, as ectopic expression of cyclin D2 is sufficient to abolish the cell cycle arrest and apoptosis induced by IL-3 withdrawal or BCR/ABL inactivation, while over-expression of $p27^{Kip1}$ can cause cell cycle arrest and apoptosis in BaF3 cells. Next, the signal pathways triggered by BCR/ABL and IL-3 to regulate cell proliferation and apoptosis *via* cyclin D2 and $p27^{Kip1}$ were investigated. The PI 3-Kinase inhibitor LY294002 blocks the ability of BCR/ABL or IL-3 to induce cyclin D2 up-regulation and $p27^{Kip1}$ down-regulation and inhibits BCR/ABL-induced entry in S phase. Ectopic expression of cyclin D2 was found to overcome the cell cycle arrest induced by inhibition of PI 3-Kinase by LY294002. The results indicate that BCR/ABL and IL-3 target cyclin D2 and $p27^{Kip1}$ to mediate cell cycle arrest and apoptosis through a pathway involving the phosphatidylinositol-3 kinase (PI 3-K).

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Declaration

The work presented in this thesis was performed entirely by myself and in no way forms part of any other thesis. This work was carried out while I was a student at the Ludwig Institute for Cancer Research at the St Mary's campus of Imperial College of Science, Technology and Medicine under the supervision of Dr. Eric Lam (Ludwig Institute) and Dr. Roger Watson (University of London).

Publications

The following publications were published during the course of the work presented in this thesis:

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Deininger MWN, Vieira SAD, **Parada Y**, Banerji L, Lam EW-F, Peters G, Mahon F-X, Köhler T, Goldman JM and Melo JV. (2001). Direct relation between bcr-abl tyrosine kinase activity and cyclin D2 expression in lymphoblasts. *Cancer Research.* 61(21):8005-13.

Abbreviations	
(-/-)	Homozygous gene knock out
Aa	aminoacid
Abl	Abelson
ALL	Acute lymphoblastic leukemia
AMP	Adenosine 5'-diphosphate
AMPS	Ammonium Persulphate
ATP	Adenosine 5'-triphosphate
BCR	Breakpoint Cluster Region
BSA	Bovine Serum Albumin
c-Abl	Cellular homologue of Abelson oncogene
CML	Chronic myelogenous leukemia
CAK	Cdk-activating kinase
cAMP	Cyclic AMP
CDK	Cyclin-dependent kinase
CIAP	Calf Intestinal alkaline phosphatase
CKI	Cyclin-dependent kinase inhibitors
Da	Dalton
dd	Double distilled
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
Dox	Doxycycline
E1A	Adenovirus early gene 1A
ECL	Enhanced Chemi-Luminescence
DHFR	Dihydrofolate reductase
EDTA	Ethylenediaminetetra-acetic acid

FACS	Fluorescence-activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
gp	Glycoprotein
GST	Glutathione-S-transferase
GTP	Guanidine Triphosphate
HDAC	Histone deacetylase
HRP	Horseradish peroxidase
IFN	Interferon
IL-3	Interleukin 3
INK4	Inhibitor of CDK4
JAK	Janus kinase
MAPK	Mitogen-activated protein kinase
M	Molar
MOPS	3-(N-Morpholino)propanesulphonic acid
NLS	Nuclear localisation sequence
NP40	Nonidet-P40
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PI 3-K	Phosphatidylinositol 3'-kinase
PKB	Protein Kinase B
RB	Retinoblastoma protein
mRNA	Messenger Ribonucleic acid

STAT	Signal transducers and activators of transcription
SV40	Simian Virus 40
TEMED	N,N,N'N'-tetramethylethylenediamine
TK	Thymidine kinase
TGF	Transforming Growth Factor
Tween-20	Polyoxyethylene-sorbitan monolaurate
V	Volt
WT	Wild-type

Chapter 1. INTRODUCTION

1.1 The eukaryotic cell cycle.

The production of two genetically identical daughters from a mother cell is known as the cell cycle and consists of three sequential processes: cell growth, DNA replication and cellular division. The cell cycle is classically divided into four phases (Figure 1.1), the most distinct being the S (Synthesis) phase where DNA replication occurs and the DNA content increases from 2N to 4N, and the cell-division phase M (Mitosis). M and S phases are separated by Gap phases, G1 before DNA replication, during which the cell doubles its mass and duplicates all of its contents, and G2 before mitosis.

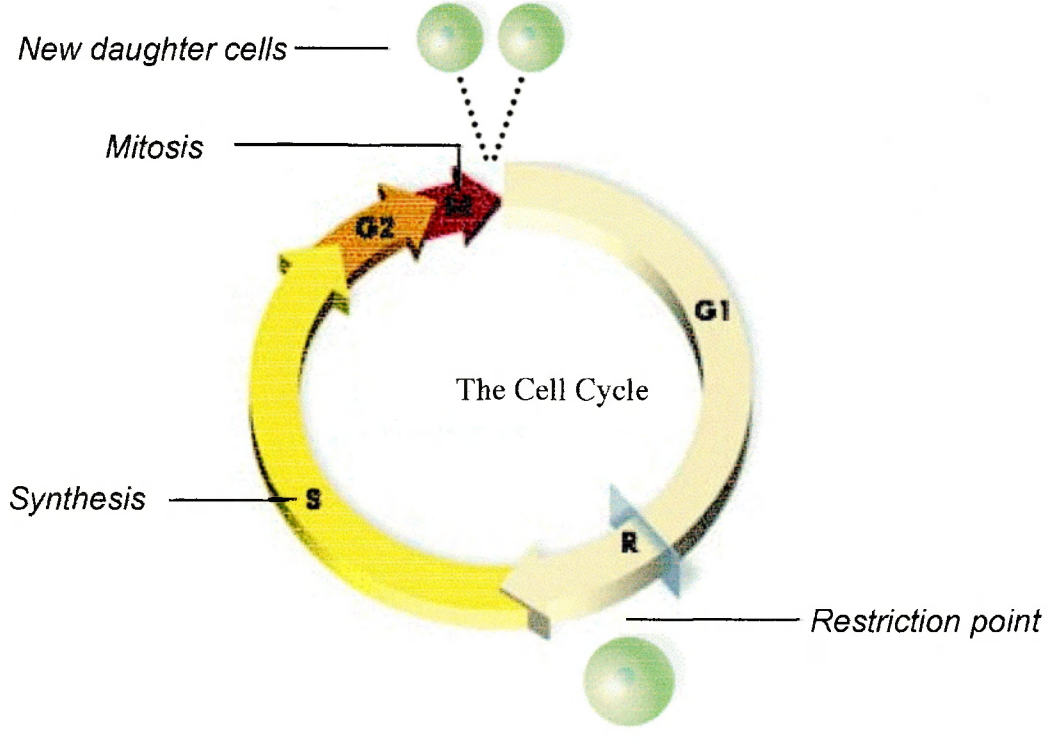

Figure 1.1. The cell cycle and its phases.

Once a cell becomes committed to divide by passing the restriction point (R), in its cycle late in Gap 1 phase (G1), it will replicate its DNA in the synthesis phase (S), proceed through the Gap 2 phase (G2), then divide in mitosis (M) and enter the G1 phase of the next cycle.

Multicellular organisms are composed of interacting communities of cells whose reproduction is tightly regulated to ensure that new cells are made only when they are needed. Therefore, it is essential to control the rate of cell proliferation and the rate of cell death. In order to do so the growth and division of each of its cells is carefully regulated and co-ordinated with its neighbours. The induction of proliferation requires the co-ordinated action of multiple intra- and extra-cellular signals.

1.1.1 The restriction point

In each division cycle, most cell types need not only to replicate their DNA, but also to grow and double their mass in order to maintain a constant size of the daughter cells. To achieve such precise co-ordination between duplicating their genome and increasing their size, cells are controlled by extracellular signals, for example, stimulation by mitogens.

The decision to either enter the S phase and divide, or remain in the G1 phase with an unduplicated genome takes place in mid-late G1 and is referred to as the restriction point (R). Whereas cells traversing the initial, pre-R, period of the G1 phase are strictly dependent on the presence of mitogenic stimuli, progression through R serves as a kind of molecular switch that results in a reduced requirement for growth factors in the post-R period of the G1, S, G2 and M phases (Reed, 1997; Solvason *et al.*, 2000). Once the restriction point is passed the cells are committed to the rest of the cell cycle. Thus, the restriction point (R) can be seen as a critical regulatory mechanism allowing a cell to monitor its environment and its own size, and an integrator and fundamental decision maker controlling the switch between alternative cellular fates such as cell division, temporal cell cycle arrest, quiescence and differentiation.

A second decision point exists prior to the onset of mitosis, at the G2/M border. The main function of the G2/M pause is to ensure that DNA replication is complete and the replicated chromosomes are aligned and ready to be separated at mitosis.

Upstream, an intricate net of cell cycle regulators integrates the signals from various sources to ensure an accurate decision about whether or not to pass the restriction points.

1.1.2 Transcription Factor E2F

The regulation of eukaryotic gene expression during development, or in response to extracellular signals, is controlled primarily at the level of transcription. Transcription is regulated in a complex manner involving the co-ordinated action of the general or basal transcription machinery and specific transcription factors.

E2F is a family of heterodimeric transcription factors each consisting of a member of the E2F family of proteins (E2F1-6) (Ginsberg *et al.*, 1994) together with one of the three known members of the DP family of co-activators (DP1-3). The E2F family includes five characterised members: E2F-1 through E2F-5, that largely act as transcriptional activators *via* a transactivation domain located at their C-terminus (La Thangue, 1996), and a sixth, isolated more recently, E2F-6 or EMA. The latter has been reported to repress transcription because it carries a DNA-binding, but not a transcriptional activation domain (Cartwright *et al.*, 1998; Morkel *et al.*, 1997). Each of the E2F and DP proteins contains a conserved DNA binding domain and dimerisation domain. The minimal DNA binding domain (DB) of E2Fs encompasses a stretch of basic residues, along with overlapping helix-loop-like and putative leucine-zipper-like domains. The basic region shows the highest level of sequence conservation between the first five members of the

family, a fact which may explain why those E2F/DPs heterodimers are able to bind *in vitro* to a similar consensus binding site on DNA with no apparent differences in affinity. This minimal DB of E2Fs requires additional flanking regions to heterodimerise with DP proteins. The first flanking region is a short one immediately adjacent to DB containing a conserved heptad repeat with a hydrophobic residue. The second one covers a conserved domain termed "the marked box" with no predictable tertiary structure (Jost *et al.*, 1996; Lees *et al.*, 1993).

The E2F responsive genes can be divided into two groups:

1. a group that includes genes directly involved in the control of cell cycle progression, for instance *E2F1, cdc2, cyclin A, D1, E, c-myc* [Stewart, 1995 #171] *and B-myb* [Lam, 1995 #172], and
2. a group that contains genes coding for proteins involved in the actual DNA and chromatin biosynthesis an d that are co-ordinately up-regulated in late G1, such as *DHFR, DNA pol(α), TK, TS, PCNA,* etc.

E2F binds to the promoters of these genes through binding sites related to the E2 sites with the consensus sequence TTT(G/C)(G/C)CG(G/C) and causes either transcriptional activation as "free" E2F or repression in the form of "complexed" E2F.

The activity of E2F/DP heterodimers is subject to several levels of control. The expression of E2F-1 to E2F-3 is regulated at transcriptional levels in a cell cycle-dependent manner, being minimal in G0 and maximal at the G1/S transition (Moberg, et al., 1996; Slansky and Farnham, 1996); in the case of the E2F-1 and E2F-2 gene promoters, this regulation is mediated by E2F-binding sites, which indicates an autoregulatory loop. Conversely E2F-4 and E2F-5 levels tend to remain constant throughout the cell cycle; however, their activity is critically dependent on regulated relocalisation to the nucleus upon interaction, either with a

DP family member containing a nuclear localisation signal (NLS), or with a pocket protein (de la Luna *et al.*, 1996; Verona *et al.*, 1997). The DP components show no significant fluctuations.

E2F/DP heterodimers interact with the pRB, p107 and p130 members of the pocket protein family. These proteins antagonise transcriptional activators of the E2F family with a specificity that is mainly determined by the E2F component (Moberg *et al.*, 1996).

A further level of control of E2F proteins activity is represented by the ubiquitin/proteasome pathway. Targeting of E2F1 for ubiquitination occurs through the carboxyl-terminal region containing the pocket protein binding domains. Because of the overlapping nature of key pRB interaction sequences with ubiquitination targeting sequences of E2F1, pRB inhibits E2F1 ubiquitination and turnover (Campanero and Flemington, 1997; Hateboer *et al.*, 1996).

1.1.3 Regulation by the pocket proteins

The three members of the pRB family (pRB, p107 and p130) are phosphoproteins, which are phosphorylated in a cell cycle dependent manner. The pRB family members can associate with E2F/DP heterodimers *via* their "pocket" domain . This association is controlled by the phosphorylation status of the pocket protein (Xiao *et al.*, 1996). Multiple sites are phosphorylated in pRB, p107 and p130. The underphosphorylated forms of pRB, p107 and p130 are capable of binding and thereby inhibiting E2F/DP heterodimers. Although the pocket proteins are similar they interact with different E2F proteins. The pRB protein has been shown to bind preferentially to E2F1, E2F2 and E2F3, while p107 and p130 bind to E2F4 and E2F5 (Moberg *et al.*, 1996). E2F6 has not been shown to bind any of the pocket

proteins. Phosphorylation of pocket proteins in mid-to-late G1 phase is functionally important because it is associated with the loss of the growth suppressive activities of the three pocket proteins. Indeed, following hyperphosphorylation of pRB, transcription of E2F-responsive genes and cell proliferation is initiated. Thus, pRB, p107 and p130 play a key role in the negative regulation of E2F activity.

The retinoblastoma tumour suppressor protein (pRB) is a rather important regulator of the G1 phase that is often disabled or lost, rather than overproduced, in cancer. The retinoblastoma gene, *Rb*, was identified over a decade ago as the first tumor suppressor (Friend *et al.*, 1986; Cavenee *et al.*, 1983). Although the gene was initially cloned as a result of its frequent mutation in the rare pediatric eye tumor, retinoblastoma, it is now thought to play a fundamental role in cellular regulation and is the target of tumorigenic mutations in many cell types. The tumor suppressor function of *Rb* was confirmed by its ability to inhibit the malignant phenotype when expressed in *Rb*-tumor cells (Huang *et al.*, 1988). Independent studies of oncogenes encoded by various small DNA tumor viruses provided the first clues that the pRB protein might be involved in the control of cellular proliferation. Expression of viral oncoproteins, such as adenovirus E1A, SV40 large T antigen or human papillomavirus E7 proteins, had been known to stimulate cell proliferation. Subsequently, these oncoproteins were found to bind directly to pRB (Dyson *et al.*, 1989b; Hu *et al.*, 1990; Ludlow *et al.*, 1989) and the regions needed for pRB association were found to overlap with the regions needed for oncogenic transformation, suggesting that viral proteins deregulated cell proliferation by binding and therefore inactivating pRB (Whyte *et al.*, 1988; DeCaprio *et al.*, 1989; Munger *et al.*, 1989).

Mutational studies of *Rb* suggested that the viral oncoproteins, above, targeted all the same functional domain of pRB consisting of two non-contiguous regions,

referred to as "the pocket" (Ewen *et al.*, 1992; Stirdivant *et al.*, 1992). Domains A and B interact with each other along an extended interdomain interface to form the central "pocket", which is critical to the tumor suppressor function of *Rb* (Chow and Dean, 1996). Viral oncoproteins contain an LXCXE motif that allows them to bind pRB at the B domain (Dyson *et al.*, 1989b; Ludlow *et al.*, 1989). However, domain A is required for domain B to assume an active conformation (Kim and Cho, 1997).

pRB operates through the modification of gene expression. pRB can repress transcription by at least two different mechanisms. First, it binds the transactivation domain of E2F blocking its ability to activate transcription (Weintraub *et al.*, 1992). Second, the pRB-E2F repressor complex that forms at promoters can actively repress transcription. pRB inhibits E2F by recruiting chromatin remodeling enzymes, including the HDACs (histone deacetylases), a family of at least seven different enzymes that remove acetyl groups from the tails of histone octamers, which appears to facilitate condensation of nucleosomes into chromatin (Brehm *et al.*, 1998). This, inhibits gene expression by blocking access of transcription factors to the promoter. Whereas pRB requires sequences in the pocket and in the carboxy-terminal region to bind and inhibit E2F, the pocket alone is sufficient for active repression when pRB is tethered directly to the promoter (Sellers *et al.*, 1995; Bremner *et al.*, 1995; Weintraub *et al.*, 1995).

Progression of a cell through G1 and S phases requires inactivation of pRB by phosphorylation, as only unphosphorylated or hypophosphorylated forms are able to associate with their targets. The hypophosphorylated forms of pRB are thought to be active, capable of binding and thereby inhibiting E2F complexes. This form of pRB is present in G0 and early and mid G1 phases. Under conditions favouring G1 this E2F-binding competent form of pRB is progressively phosphorylated until it is inactivated in the late G1 phase, resulting in derepression of E2F and

other pRB-bound transcription factors, culminating in the activation of the transcription of S-phase genes (Figure 1.2) (Dyson, 1998; Weinberg, 1995). At this point, the cell is irreversibly commited to enter the S phase. pRB retains this hyperphosphorylated state throughout the remainder of the cell cycle until passage through mitosis, when the protein is quickly dephosphorylated. Removal of these phosphates appears to be carried out by a multimeric complex of protein phosphatase type 1 (PP1) and noncatalytic regulatory subunits at the completion of mitosis (Tamrakar *et al.*, 2000). Thus, the hyperphosphorylated (inactive) form predominates in proliferating cells, whereas the hypophosphorylated (active) form is generally more abundant in quiescent or differentiating cells.

A variety of physiological growth-inhibiting signals prevent pRB phosphorylation and so block cell cycle progression. These signals, which include TGF-β, cyclic AMP and contact inhibition, activate the CKIs, that then associate with either a CDK or a cyclin-CDK complex, blocking its function, and therefore preventing pRB phosphorylation (discussed in the next section) (Hannon and Beach, 1994, Kato *et al.*, 1994, Dulic *et al.*, 1994).

p107 and p130 were originally identified as targets of the transforming domains of viral oncoproteins encoded by small DNA tumor viruses (Dyson *et al.*, 1989a; Ewen *et al.*, 1989). p107 and p130 are structurally very similar to pRB with the greatest homology lying in the pocket, hence they also can inhibit E2F-responsive promoters, recruit HDAC to the pocket, actively repress transcription and arrest the growth of cells when they are overexpressed (Paggi *et al.*, 1996; Ferreira *et al.*, 1998).

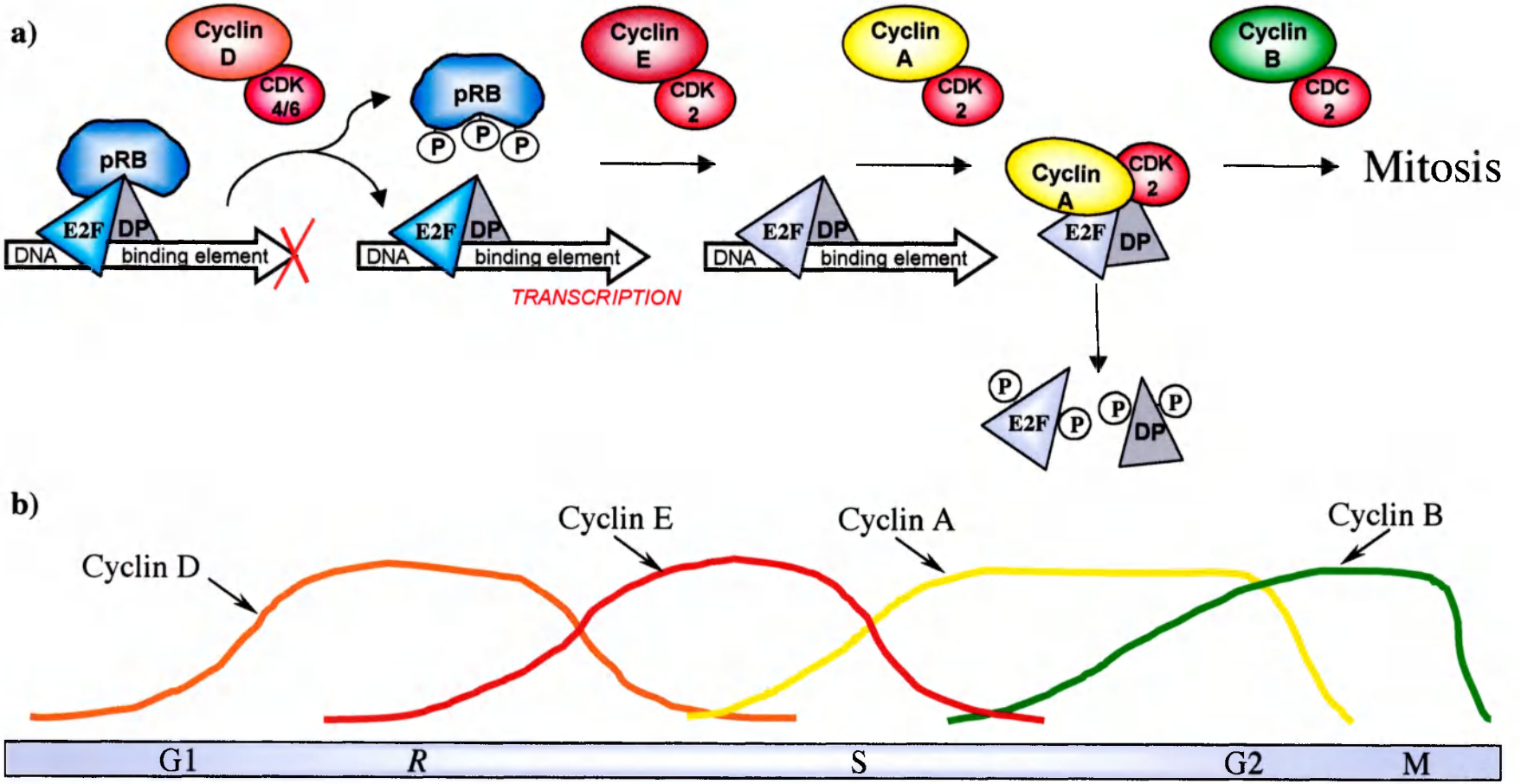

Figure 1.2. Cell cycle-dependent changes of pRB-E2F complexes on promoters.

a) In G1, hyposphorylated pRB binds and inhibits E2F1-3/DP complexes as soon as these factors are synthetised. As cells progress through G1, the cyclin D-associated kinases phosphorylate progressively pRB, resulting in the release of E2F/DP and the transcriptional activation of E2F-responsive genes. As cells move into the S phase, E2F forms stable complex with cyclin A-cdk2. The bound enzyme phosphorylates E2F and DP suppressing the E2F DNA-binding activity and E2F-activated transcription in S.
b) Cyclins are regulated in a cell cycle-dependent way.

There are significant differences between the pocket proteins: for example, the spacer sequences between domains A and B are longer in both p107 and p130 than in the analogous region in pRB and present little homology. The p107 and p130 spacer regions include a high-affinity binding site for cyclin A/CDK2 and cyclin E/CDK2, which allows them to form stable complexes. In addition, p107 and p130 each contain insertions within the B domain that are missing from pRB (Ewen *et al.*, 1992; Hannon *et al.*, 1993; Li *et al.*, 1993).

Whilst the levels of pRB are quite constant during the cell cycle and in quiescent cells, levels of p130 and p107 change dramatically depending on the stage of the cell cycle. p130 is highly expressed in both quiescent and differentiated cells, and its levels drop rapidly when quiescent cells are stimulated to enter the cell cycle (Cobrinik *et al.*, 1993). In contrast, p107 levels are generally quite low in terminally differentiated cells and they rise when quiescent cells are stimulated to proliferate (Raschella *et al.*, 1997; Shin *et al.*, 1995).

Biochemical and genetic studies suggest that the pocket proteins have partially overlapping as well as distinct functions in cell cycle control and mouse development (Mulligan and Jacks, 1998). Mice lacking functional pRB died during embryogenesis, exhibiting defective erythropoiesis as well as excessive proliferation and cell death in the liver, lens and nervous system (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Conversely, animals lacking p107 or p130 developed normally (Cobrinik *et al.*, 1996). However, the interbreeding of *p130* and *p107*, *Rb* and *p107* or *Rb* and *p130* mutant mouse strains has revealed significant functional overlap within the gene family. Mice deficient in p130 and p107 exhibit excessive chondrocyte proliferation, bone defects and rapid postnatal death (Cobrinik *et al.*, 1996). p107 deficiency accelerates the *Rb-/-* embryonic lethality by 2 days (Lee *et al.*, 1996). These results suggest that, although having distinct roles in tumour suppresion and development, in the absence of a particular

pocket protein some mechanisms of compensation exist by the remaining proteins.

1.1.4 Regulation of pRB activity through phosphorylation by CDKs

Nearly all the phosphorylation sites identified in the pocket proteins fall within the consensus site of phosphorylation by Cyclin-Dependent Kinases (CDKs). Four of the CDKs function in cell cycle progression: CDC2 (CDK1), CDK2, CDK4 and CDK6. The activity of the CDKs is regulated by multiple mechanisms, including protein-protein interactions, protein degradation, transcriptional control, subcellular localisation, and multiple phosphorylations (Morgan, 1995; Solomon and Kaldis, 1998).

Cellular CDK levels tend to remain in constant excess throughout the cell cycle, but their catalytic activity requires activation by association with one or more of the 16 regulatory cyclin subunits and by their phosphorylation or dephosphorylation (Johnson and Walker, 1999; Pines, 1997; Sherr, 1993). Unlike CDKs, cyclins are regulated at the transcriptional level in a cell cycle-dependent way, as represented in Figure 1.2b, and therefore their expression is rate limiting for progression through the different stages of the cell cycle. Cyclin levels are also modulated by protein turnover. The ubiquitin-mediated proteolysis of cyclins ensures the irreversible and abrupt inactivation of the associated CDK. The cyclin family is divided into two main classes:

1. The 'G1 cyclins' include cyclins D1-3, and E, and their accumulation is rate-limiting for progression from the G1 to S phase.
2. The 'mitotic or G2 cyclins', which include cyclin A and cyclin B, are involved in the control of G2/M transition and mitosis.

Once assembled, the CDK/cyclin complex is activated by dephosphorylation of inhibitory sites (Thr-14 and Tyr-15) by the dual-specificity phosphatase CDC25, and phosphorylation on a single threonine residue (Thr-160 in human CDK2, Thr-161 in CDC2)) (Figure 1.3) (Morgan, 1995). The phosphorylation is mediated by the enzyme complex CDK-activating kinase (CAK), which is itself a multimeric enzyme composed of a CDK subunit (MO15 or CDK7) and a cyclin partner (cyclin H) (Harper and Elledge, 1998). CAK appears to be present constantly during the cell cycle, suggesting that mitogenic signals are not required for its induction, but certain CDK inhibitors can prevent CAK from accessing its substrates, thereby preventing the formation of enzymatically active cyclin/CDK complexes (Kato *et al.*, 1994). It has been proposed that phosphorylation of the Thr residue by CAK induces a conformational change within the CDK/cyclin complex to fully expose the catalytic cleft in order that substrates may bind (Jeffrey *et al.*, 1995; Russo *et al.*, 1996).

The first cyclin/CDK to be activated after growth factor stimulation contains a D-type cyclin (D1, D2, D3) and either CDK4 or CDK6 (Figure 1.2a), and has a distinct substrate preference for the retinoblastoma protein (RB). Unlike other members of the cyclin family, cyclins D1, D2 and D3 have unique cell- and tissue-specific patterns of expression (Sherr, 1993). After mitogen withdrawal, D-type cyclins are rapidly degraded. Cyclin E is synthesised later than D-type cyclins, with expression levels peaking at the G1/S boundary, and associates with CDK2; the resultant complex has a broader specificity, phosphorylating histone H1, pRB and the CDK inhibitor $p27^{Kip1}$.

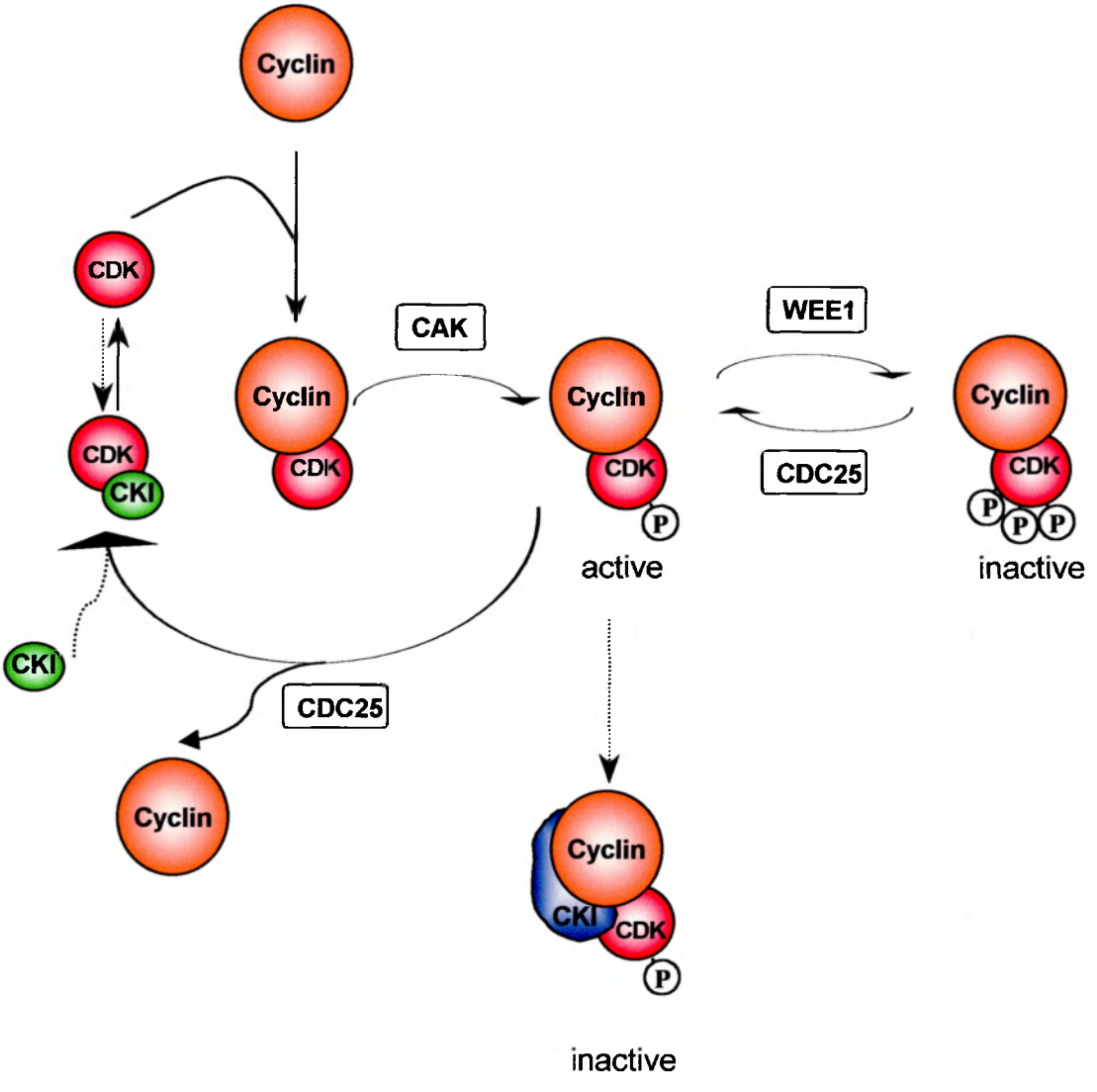

Figure 1.3. Model for CDK regulation. The activation of the CDKs is determined by their post-translational modifications and by the association with a cyclin.

Upon association with a cyclin, CDK is activated by phosphorylation by the CDK-activating kinase (CAK) on a single residue. The catalytic activity is inhibited by further phosphorylation by Wee1 on two residues; dephosphorylation by CDC25 and removal of the cyclin subunit; or binding by a CDK inhibitor (CKI) (dashed arrows)

Once the cells enter into the S phase, cyclin E is degraded and CDK2 forms complexes with cyclin A. The last complex to be formed comprises CDC2 and cyclin B and triggers the entry into mitosis. In conclusion, D-type cyclins are responsible, together with their partner kinases CDK4/6, for the phosphorylation of pocket proteins in G1, resulting in abrogation of the interaction of the pocket proteins with E2F/DP complexes. Upregulation of cyclin D seems to be more important for the G1/S transition, whereas cyclin E seems more important for S phase entry. Cyclin A helps to maintain pRB in hyperphosphorylated forms until exit from mitosis.

Both the D-type cyclins and their partner kinases, CDK4/6, have proto-oncogenic properties, and their activity is carefully regulated at multiple levels. Four major mechanisms have been identified to inactivate CDK-cyclin complexes:

1. Phosphorylation at two sites near the amino terminus (Thr-14 and Tyr-15 in human CDC2). Phosphorylation of these residues is important for the control of CDC2 activity and abrupt dephosphorylation at these sites occurs at mitosis.
2. Removal of cyclin.
3. Dephosphorylation of the Thr 160/161 residue, by the phosphatase CDC25.
4. Inhibition by inhibitory subunits.

1.1.5 Regulation of CDK activity during the cell cycle.

The catalytic activity of the cyclin/CDK complexes is controlled by several mechanisms, one of which involves a family of low molecular weight inhibitors. The CDK inhibitors (CKIs) represent a growing superfamily of negative regulators that help to mediate temporary arrest or permanent withdrawal from the

cell cycle, either in response to anti-mitogenic or genotoxic stimuli, or as part of programmes of terminal differentiation. Thus, they play a role in development, checkpoint control and tumour suppression. In mammalian cells, two classes of CKIs have been identified: the INK4 (inhibitors of CDK4) class and the CIP/KIP class (Sherr and Roberts, 1999). The activity of members of the INK4 subfamily, $p15^{INK4b}$ (Hannon and Beach, 1994), $p16^{INK4a}$ (Serrano *et al.*, 1993), $p18^{INK4c}$ and $p19^{INK4d}$, is particularly linked with pRB as their ability to inhibit the cell cycle depends on functional pRB (Lukas *et al.*, 1995; Medema *et al.*, 1995; Sherr and Roberts, 1995). They specifically inhibit the CDK4/6 dependent-kinases (Roussel, 1999). $p16^{INK4a}$ has been shown to play an important role in cellular senescence, and its loss results in cell immortalisation (Serrano *et al.*, 1996).

The members of the CIP/KIP subfamily of CKIs, $p21^{Cip1}$, $p27^{kip1}$ and $p57^{Kip2}$, contain characterisitic motifs within their amino-terminal moieties that enable them to bind and therefore inhibit most cyclin-CDK complexes (Harper, 1997). $p21^{Cip1}$ was the first mammalian CKI to be identified (Harper *et al.*, 1993; Xiong *et al.*, 1993). It is well established that $p21^{Cip1}$ consists of a least two functional domains that bind to CDK/cyclins and proliferating cell nuclear antigen (PCNA) (Podust *et al.*, 1995; Xiong *et al.*, 1992; Zhang *et al.*, 1993). In addition to interacting with the CDK, the independent binding of $p21^{Cip1}$ to PCNA seems to block its ability to activate DNA pol δ, inhibiting DNA replication (Chen *et al.*, 1995; Nakanishi *et al.*, 1995). $p21^{Cip1}$ is also involved in the p53-mediated G1 arrest cellular response to DNA damage (Sherr and Roberts, 1995). Mice nullizygous for $p21^{Cip1}$ develop normally, but have a more relaxed response of their cells to DNA damage, consistent with a role of $p21^{Cip1}$ in G1 phase checkpoint control (Brugarolas *et al*, 1995; Ando *et al.*, 2001).

$p27^{Kip1}$is a widely expressed inhibitor of the essential cell cycle kinase CDK2, which regulates entry into the S phase (Sherr and Roberts, 1999). $p27^{Kip1}$ was first

identified as an inhibitor of cyclin E/CDK2 (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994). High levels of $p27^{Kip1}$ inhibit the activity of the CDK2/cyclin E complex, prevent phosphorylation of critical target molecules for initiation of the S phase, including pRB, and induce an arrest in G1 (Hsieh *et al.*, 2000). In normal cells, progression through G1/S phase requires that $p27^{Kip1}$ be displaced from CDK2, either by sequestration in cyclin D/CDK4 complexes, or by down-regulation of the protein through multiple mechanisms. Many antiproliferative signals lead to $p27^{Kip1}$ accumulation, including mitogen/cytokine withdrawal, cell-cell contact and agents such as cAMP and rapamycin (Sherr and Roberts, 1995). The role of $p27^{Kip1}$ seems to be very important in regulating cell proliferation as the $p27^{Kip1}$ knockout mouse exhibits gigantism, female sterility and increased tumourigenesis (Fero *et al.*, 1996; Nakayama *et al.*, 1996).

The cellular threshold levels of CKIs appear to be carefully controlled by a variety of mechanisms, including transcriptional regulation, post-transcriptional regulation, sequestration in cellular compartments and degradation. For $p21^{Cip1}$, a major mode of regulation is transcriptional, induced by p53, a transcriptional regulator that mediates cell-cycle arrest following DNA damage and in senescence (Dulic *et al.*, 1994). Transcriptional control may also be important for $p15^{INK4b}$, whose expression in human keratinocytes is greatly enhanced by treatment with the negative growth factor TGF-β (Hannon and Beach, 1994). $p27^{Kip1}$ expression has been shown to be regulated at various levels. It occurs at both transcriptional and translational levels. $p27^{Kip1}$ may be degraded by the ubiquitin/proteasome pathway (Esposito *et al.*, 1997; Hengst and Reed, 1996). $p27^{Kip1}$ is also regulated by phosphorylation (Vlach *et al.*, 1997). The most recently proposed mechanism of $p27^{Kip1}$ regulation is subcellular compartmentalisation (Orend *et al.*, 1998). $p27^{Kip1}$ appears to interact with its targets in the cell nucleus, and mislocalisation of $p27^{Kip1}$ in the cytoplasm might

inactivate $p27^{Kip1}$ by sequestering it away from the relevant cellular targets (Depoortere *et al.*, 2000; Liu *et al.*, 2000). These multiple control mechanisms seem justified given that elevated levels of CKIs from either subfamily can cause G1 phase arrest, suggesting that these integral elements of the cell cycle machinery exert a significant influence on the G1 phase checkpoint control.

1.1.6. Cell cycle arrest and apoptosis

Control mechanisms are required to guarantee that all the necessary preparations are completed in the correct sequence before cell division occurs. Of the processes that must be given adequate time for completion, DNA replication is one of the most critical (Hartwell and Weinert, 1989; Murray, 1992). DNA damage can occur by exposure to mutagens such as ultraviolet light, x-rays, chemotherapeutic drugs or increased levels of oxidants within the cell, or due to replication errors. To protect themselves from accumulating DNA damage, mammalian cells possess mechanisms which ensure that deleterious mutations are removed from the population.

The first one is the arrest in G1 phase of the cell cycle. In early G1 phase, the CDKs of the cell-cycle control system are reduced to an inactive state by several molecular braking mechanisms. In the absence of the appropriate signals to proliferate, these brakes are held in place and CDK inhibition is maintained, prolonging G1 phase. When proliferation signals appear, the brakes are released and CDK activation occurs, leading to a new round of cell division. However, damaged cells reversibly arrest their cycle at checkpoints, allowing time for the necessary DNA repair.

The second mechanism is referred to as apoptosis. If the damage is too great, the cell may activate apoptotic pathways, leading to its death. Non-necrotic cell death

has been known of for at least fifty years (Glucksmann, 1965), and in 1972, the term apoptosis was coined to describe this form of programmed cell death (Kerr *et al.*, 1972). Apoptosis describes the process by which cells are removed under normal conditions when they reach the end of their life span, are damaged, or superfluous. Morphologically, in cells undergoing apoptosis, there is ruffling and condensation of the plasma and nuclear membranes, and subsequently, aggregation of nuclear chromatin (Lowe and Lin, 2000). The cell shrinks and then fragments into a cluster of membrane-enclosed "apoptotic bodies" that are rapidly ingested by adjacent macrophages (Wyllie *et al.*, 1980). The hallmark of the end stage of apoptosis is the endonuclease cleavage of DNA in the internucleosomal linker regions, yielding 180 base-pair fragments. Apoptosis is a general tissue phenomenon necessary for development and homeostasis: elimination of redundant cells during embryogenesis, cell atrophy upon endocrine withdrawal or loss of essential growth factors or cytokines, tissue remodelling and repair, and removal of cells that have sustained genotoxic damage.

Defects in these processes can lead to excess cell proliferation and, under some conditions, cause cancer.

1.2 Haematopoiesis.

The production of mature blood cells, haematopoiesis, occurs predominantly in the adult bone marrow. Differentiation of a limited population of self-renewing pluripotent stem cells results in the generation of distinct lineage-committed myeloid or lymphoid progenitor cells (Figure 1.4); these cells can be further induced to differentiate. The myeloid progenitor is the precursor of the granulocytes, monocytes, megakaryocytes and erythrocytes, whilst the common lymphoid progenitor gives rise to the lymphocytes, which are responsible for the specificity of the immunoresponse. There are two major types of lymphocytes: B lymphocytes, which when activated differentiate into plasma cells that secrete antibodies, and T lymphocytes, which exert cell-mediated responses.

1.2.1 Regulation of haematopoiesis.

Early research in haematopoiesis demonstrated that haematopoietic cells could not be maintained in culture unless certain viability factors or viability factor-producing cells were provided (Sachs, 1987; Sachs, 1988; Sachs, 1996). Cell culture supernatants were used to isolate, characterise and clone the genes coding for a large family of soluble polypeptides referred to as haematopoietic cytokines. Cytokines are a unique family of growth factors able to strictly control the proliferation, differentiation, survival and effector functions of haematopoietic progenitor cells. Secreted primarily from leukocytes, cytokines stimulate both the humoral and cellular immune responses, as well as the activation of phagocytic cells. Cytokines that are secreted from lymphocytes are termed lymphokines, whereas those secreted by monocytes or macrophages are termed monokines.

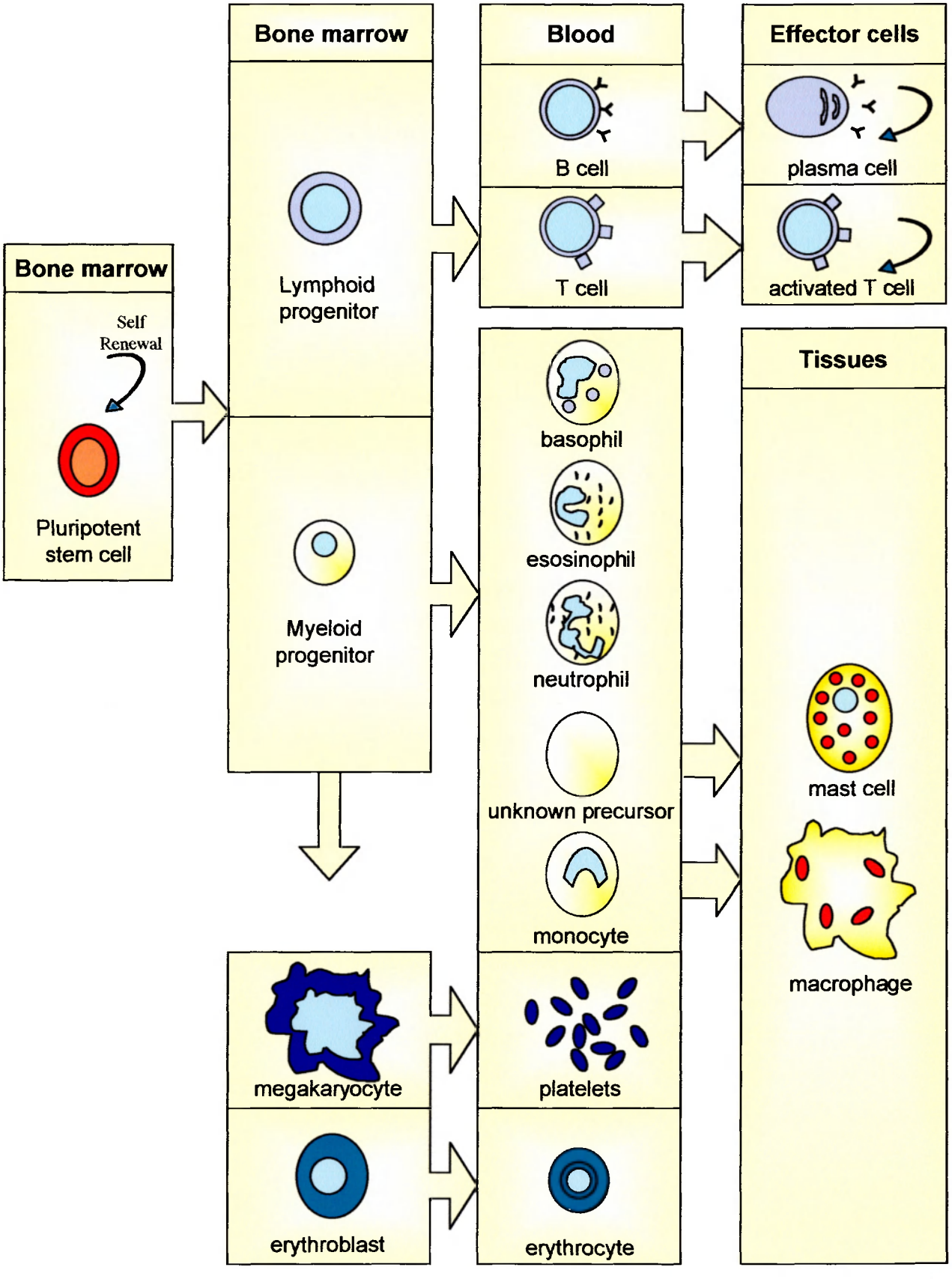

Figure 1.4. Schematic representation of haematopoiesis

Originally, the cytokines were named according to their function (like the T cell growth factor, now called IL-2), but then the pleotropy of cytokines was observed, making function specific names confusing. After more and more cytokines were identified, and in order to avoid confusion, immunologists started naming some of the lymphokines 'interleukins' (ILs), since they are not only secreted by leukocytes but also able to affect the cellular responses of leukocytes, and numbering them as they were found. A sample of some cytokines and their functions is given in Table 1, below.

Colony stimulating factors (CSFs) are cytokines that stimulate the proliferation of specific pluripotent stem cells of the bone marrow in adults. Granulocyte-CSF (G-CSF) is specific for proliferative effects on cells of the granulocyte lineage. Macrophage-CSF (M-CSF) is specific for cells of the macrophage lineage. Granulocyte-macrophage-CSF (GM-CSF) has proliferative effects on both classes of lymphoid cells. Erythropoietin (Epo) is also considered a CSF as well as a growth factor, since it stimulates the proliferation of erythrocyte colony-forming units.

Other sub-groups of the cytokines are the tumour necrosis factors (TNFs), Interferons (IFNs) and the Transforming Growth Factors (TGFs).

Cytokines bind to their cognate receptors and mediate intracellular signal transduction events that result in the modulation of gene expression (Mannoni, 1993). One of the characteristic features of cytokines is their functional pleiotropy, i.e. a particular cytokine can exhibit a wide variety of biological functions on various tissues and cells. (Reddy *et al.*, 2000). Cytokines also function in a redundant manner and several different cytokines can exert similar and overlapping functions on the same cell type. Such functional pleiotropy and redundancy, initially thought to be a consequence of the structural characteristics

Cytokine	Principle Source	Principle activities
IL-1	Macrophage and other antigen presenting cells (APCs)	T,B cell activation, fever, inflammation, acute phase response, haematopoiesis
IL-2	T cells	T cell proliferation
IL-3	**T cells**	**Growth of haematopoietic progenitor cells**
IL-4	T cells	B cell growth and differentiation
IL-5	T cells	B cell, eosinophil growth
IL-6	Macrophages, T cells	B cell stimulation, inflammation
IL-7	Stromal cells	Early B and T cell differentiation
IL-8	Macrophages	Neutrophil (PMN) attraction
IL-9	T cells	Mitogen
IL-10	T cells	Inhibits Th1 cytokine production, B cell proliferation, antibody production, cellular immunity suppression, mast cell growth
IL-11	Bone marrow stroma	Synergisitc haematopoietic and thrombopoietic effects
IL-12	APC	Stimulates T, NK cells
IL-13	T cells	B cell growth and differentiation
IL-14	Dendritic cells,T cells	B cell memory
IL-15	T cells	T cell proliferation
IFN-α	Macrophages, neutrophils and some somatic cells	Anti-viral
IFN-β	Macrophages, neutrophils and some somatic cells	Anti-viral
IFN-γ	T, NK cells	Inflammation, macrophage activation
TGF-β	Macrophages, lymphocytes	Depends on target
TNF-α	Macrophage	Inflammation; tumor killing
TNF-β	T cells	Inflammation; tumor killing, enhance phagocytosis

Table 1. Properties of some Cytokines.

of cytokines themselves, is now regarded to be a reflection of similarities and differences in the proteins interacting with cytokine receptors.

Most cytokine receptors consist of a multisubunit protein complex: a unique and specific ligand binding subunit, and a ligand transducing subunit, which may be structurally similar to other members of the cytokine receptor superfamily (Onishi *et al.*, 1998). The signal transducing subunit is responsible for propagation of the signals to downstream target proteins upon cytokine binding. It recruits cytoplasmic proteins, which leads to changes in protein-protein interactions as well as changes in the phosphorylation status of several proteins (Stahl and Yancopoulos, 1993).

The cytokine receptor family has been sub-divided based on the characteristic structural motifs in their extracellular domains. The main sub-types include:

1 The gp130 family, as well as receptors for IL-6, IL-11, cardiotropin-1 (CT), ciliary neurotrophic factor (CNTF) and oncostatin M (Sanchez-Cuenca *et al.*, 1999).

2 The IL-2 receptor family, comprising the receptors for IL-2, IL-7, IL-9 and IL-15.

3 The growth hormone (GH) family, including receptors for growth hormone, prolactin, erythropoietin, GCSF and thrombopoietin.

4 The interferon (IFN) family, together with the IFN-α and -γ and the IL-10 receptor.

5 The gp140 family, including the receptors for IL-3, IL-5 and GM-CSF.

The network of growth stimulatory and inhibitory cytokines that regulates the proliferation of haematopoietic cells acts primarily in G0/G1. After each round of replication of specific lineage cells, exposure to an adequate level of lineage-

specific growth factors is required, without which the cells rapidly arrest in G1 and undergo apoptosis, a genetic program of cellular self-destruction (Dexter and Heyworth, 1994). Apoptosis is widely recognised to play a role in both the physiology and pathology of the immune system as crucial as cell division or differentiation (Allen *et al.*, 1993). For example, apoptosis is a fundamental feature during the development of the lymphocyte repertoire, being intimately involved in the negative and positive selection of T and B lymphocytes bearing antigen receptors. For these populations, dysregulation of the selection mechanisms could result in a number of disease states: deletion of clones essential to the immune response may lead to immunodeficiency; production of lymphocytes bearing antigen receptor for self would encourage autoimmunity, while overproduction of lymphocyte clones could predispose to malignancy (Squier *et al.*, 1995; Straus *et al.*, 2001). There are two classes of interaction by which apoptosis can be induced. First, by a particular stimulus, such as engagement of the T-cell receptor or the Fas/Apo-1 antigen (Mapara *et al.*, 1993; Trauth *et al.*, 1989). Second, from removal of a stimulus, such as Interleukins, which normally suppresses apoptosis (Duke and Cohen, 1986; Williams *et al.*, 1990).

In addition to soluble signalling molecules, specific intracellular contacts regulate the haematopoiesis. However, the role of the cytokines is crucial in haematopoiesis. Myeloid malignancies are often characterised by the arrest of maturation and cytokine-independent proliferation of the myeloid progenitors.

1.2.2 Interleukin-3 (IL-3) and its receptor (IL-3R)

Interleukins are growth factors targeted to cells of haematopoietic origin. The list of identified interleukins grows continuously with the total number of individual activities now at 23 (Oppmann *et al.*, 2000; Wiekowski *et al.*, 2001).

The cytokine interleukin-3 (IL-3), also referred as multi-CSF, is a 28kDa glycoprotein secreted by $CD4^+$ T cells. IL-3 is an important regulator of haematopoiesis modulating the proliferation, differentiation and survival of various haematopoietic cell lineages and their precursors, including stem cells, neutrophils, eosinophils, macrophages, etc. (Pierce, 1989).

The IL-3 receptor (IL-3R) consists of an α-subunit of 60-70 kDa and a β-subunit of 130-140 kDa (Miyajima *et al.*, 1992). Both subunits contain the extracellular conserved motifs found in the cytokine receptor superfamily, whilst the cytoplasmic domains show little similarity with other cytokine receptors and lack detectable catalytic domains, such as the tyrosine kinase domains. However, previous evidence suggests that IL3 signal transduction involves tyrosine phosphorylation (Kanakura *et al.*, 1990).

The common beta subunit (β_c) on the IL-3 receptor is shared by the receptors for IL-5 and GM-CSF, and its cytoplasmic domain can be divided into two distinctive functional domains: a membrane proximal portion responsible for the binding to multiple signal-transducing proteins such as Janus kinases (JAKs), signal transducers and activators of transcription (STATs), phosphatidylinositol-3 kinase (PI 3-K), c-Src and Vav (Woodcock *et al.*, 1996; Yoshimura *et al.*, 1996), and a membrane distal domain involved in the growth inhibition that can be induced by some of the cytokines. The α chain confers the specificity of the binding to a cytokine through its extracellular ligand-binding domain.

Three different pathways (Figure 1.5) have been reported to be predominantly responsible for most of the cellular processes induced by IL-3:

1. JAK/STAT. This important pathway in haematopoietic growth factor signalling involves activation of the non-receptor Janus family kinases (JAKs), consisting of four members: JAK1, JAK2, JAK3 and TYK2. STATs proteins are a unique family of transcription factors initially identified in interferon signalling. Upon cytokine binding, the JAKs are activated by trans-phosphorylation of two-receptor-bound JAK molecules and phosphorylate a number of substrates including the cytokine receptor. The cytokine receptor provides docking sites for a variety of Src homology 2 (SH-2) domain-containing proteins, including the STATs. Once phosphorylated the STATs dimerise, migrate into the nucleus and regulate gene transcription (Ihle *et al.*, 1998). IL-3 activation of haematopoietic cells appears to result predominantly in the activation of JAK-2, although JAK-1 and TYK-2 have also been found to be activated in certain cell systems (Chaturvedi *et al.*, 1997; Liu *et al.*, 1999). Similarly, although activation of STAT1, STAT3 and STAT6 has been observed, STAT5 seems to be the most predominant STAT activated by the IL-3 receptor (Chin *et al.*, 1996). However, over the past few years, increasing evidence has accumulated which indicates that at least some of the STAT protein activation may be mediated by members of the Src family following cytokine stimulation (Reddy *et al.*, 2000). This pathway seems to play a decisive role in cell differentiation (Parganas et *al.*, 1998; Ihle, 1995) and to contribute to survival to a lesser extent.

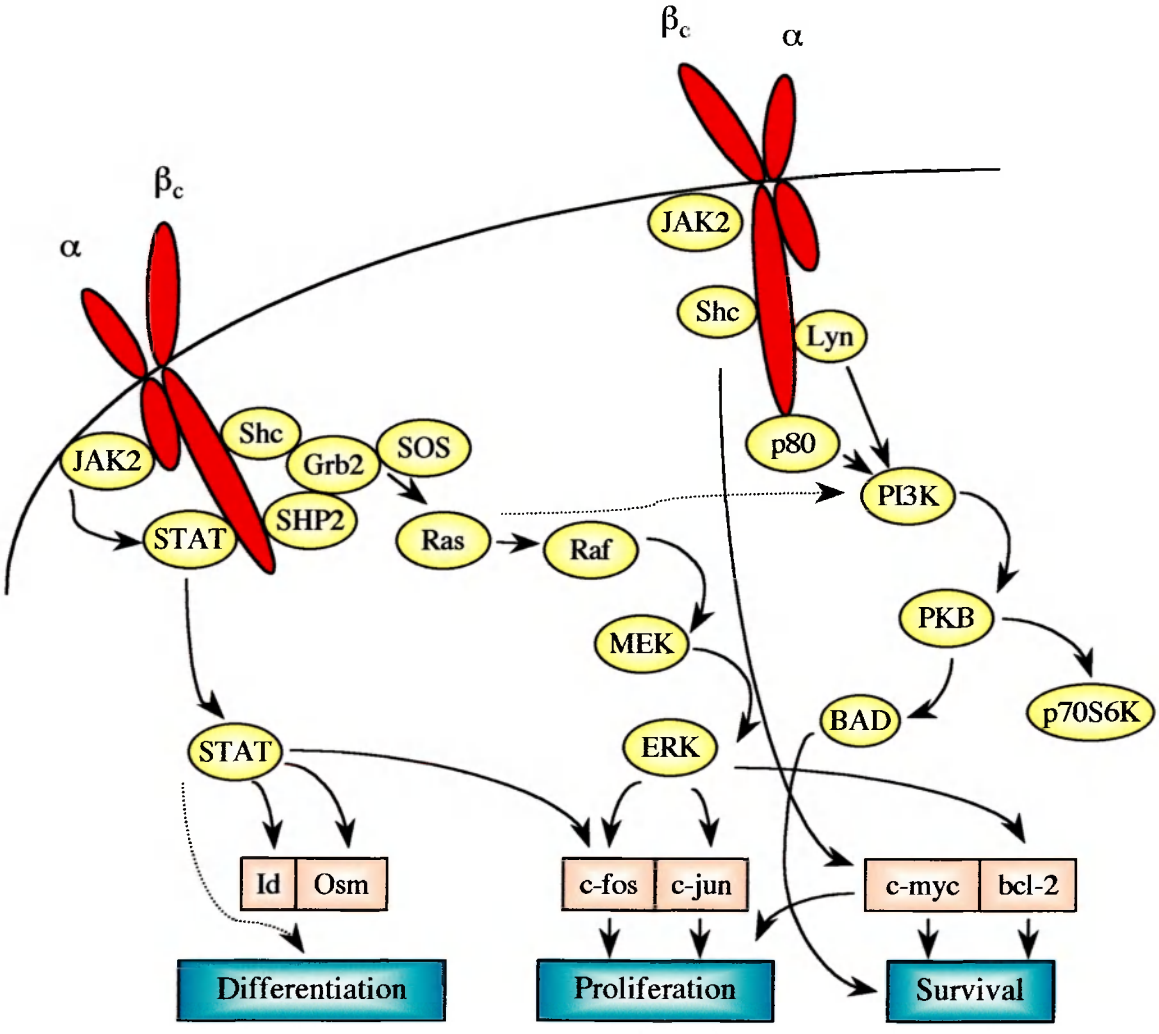

Figure 1.5. Schematic representation of signal transduction pathways activated by IL-3.

Receptors activation leads to the activation of multiple cytoplasmic signalling molecules (oval boxes). These pathways eventually lead to altered gene transcription (rectangles) or directly contribute to function, such as differentiation, proliferation and survival. Dashed lines represent connections to be demonstrated (from de Groot RP et *al.*, 2000)

2. MAPKinase. Activation of this pathway is a common event upon stimulation of haematopoieitc growth factor receptors, and in cells transformed with oncogenic tyrosine kinases. Ras belongs to the large Ras superfamily of monomeric GTPases. Downstream effectors of Ras include serine and threonine kinases, such as Raf or extracellular signal-regulated kinase (ERK). The activation of Ras can be strictly regulated by the GDP/GTP nucleotide exchange factor SOS or Ras-GAP, a Ras/GTPase activating protein. A critical event in Ras activation is the relocalisation of SOS through the adapter proteins GRB2 and Shc. Activation of the MAPKinase ERK by IL-3 is expected to occur through activation of Ras and c-Raf (Coffer et *al.*, 1998; Satoh et *al.*, 1991). This activation will result in enhanced transcription of c-*fos* and c-*jun* and might contribute to IL-3-induced proliferation (de Groot *et al.*, 1997; Terada *et al.*, 1997). Dominant-negative Ras inhibited the IL-3 induced activation of Raf and ERK and abolished almost completely c-fos expression, but IL-3 dependent proliferation and viability were not altered. In contrast, a constitutively active Ras mutant prevented unstimulated IL-3 dependent cells from apoptosis (Terada *et al.*, 1995). Studies suggest that IL-3-induced Ras-Raf-ERK activation plays an important role targeting the anti-apoptotic proteins bcl-2 and c-Myc (Kinoshita *et al.*, 1995).

3. PI 3-Kinase. Phosphatidylinositol-3 kinase is a heterodimer composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit, and is found in cellular complexes with activated growth factor receptors and oncogene protein tyrosine kinases. It catalyses the addition of a phosphate molecule specifically to the 3-position of the inositol ring of phosphoinositides, producing mainly PI-(3,4)-bisphosphate and PI-(3,4,5)-triphosphate, which function as second messengers (Rameh and Cantley, 1999). A role for PI 3-K

in signal transduction pathways and biological functions, such as reduced apoptosis, mitogenesis, integrin activation and membrane traffic have been established for various systems (Klippel *et al.*, 1996).

One of the major functions of IL-3 is the inhibition of apoptosis in the target cells. Recent advances suggest that the PI 3-K/AKT pathway contributes to survival induced by IL-3 (Coffer et *al.*, 1998). Particularly, the availability of specific inhibitors, such as LY294002 and wortmannin opened the way to more extensive studies on this pathway. Indeed, treatment with both inhibitors on BaF3 and 32D3 cells completely inhibited cellular survival induced by IL-3. Moreover, overexpression of active PKB/AKT, a downstream effector of the PI 3-K, results in IL-3-independent survival of these cells and promotes the expression of the anti-apoptotic c-*myc* and *bcl*-2 genes (Ahmed *et al.*, 1997; Songyang *et al.*, 1997). A major target for PKB/AKT was shown to be the pro-apoptotic bcl-2 family member BAD, which is phosphorylated by PKB/AKT at the same residues that are phosphorylated in response to IL-3 and that block its pro-apoptotic activity (Datta *et al.*, 1997; del Peso *et al.*, 1997).

1.3 Chronic myelogenous leukemia (CML)

Whereas the growth of normal cells is carefully regulated to meet the needs of the whole organism, one of the defining features of cancer cells is that they respond abnormally to the control mechanisms that regulate the division of normal cells. As a result cancer cells replicate continuously, ultimately interfering with the function of normal tissues. This ability is often conferred by altered genes that mimic the growth factor exposed state or suppress apoptosis and hence lead to unregulated proliferation. This set of oncogenes are mutant versions of normal cellular "proto-oncogenes". Proto-oncogenes regulate positively cellular growth in response to stimulatory signals. Once activated in cancer, proto-oncogenes are termed oncogenes, and act in a dominant fashion through a gain of function to promote cell growth. The process of activation of proto-oncogenes to oncogenes can include retroviral transduction or retroviral integration, point mutations, insertion mutations, gene amplification, chromosomal translocation and/or protein-protein interactions. Examples of oncogenes include the *Ras* family (Radhika and Dhanasekaran, 2001; Wyllie *et al.*, 1987), the *Myc* transcription factor (Lee and Reddy, 1999) and the non-receptor tyrosine-specific protein kinase ABL. In contrast, tumor suppressor genes repress cell growth in response to inhibitory signals. Defects in tumour suppressor genes involve the loss of gene function by mutation or inactivation. Some well-known tumour suppressors are the INK4 CDKIs (Sherr and Roberts, 1999) and *Rb* (Dyson, 1998). The identification of the abnormalities that result in cancerous growth can lead to the understanding of the mechanisms that control normal growth and development.

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterised by a clonal expansion of progenitor cells. As a result, patients have high leukocyte counts due to accumulation in the blood of immature cells of the granulocytic lineage. The annual incidence is approximately one to two cases per

100,000 per year, increases with age, with a male:female ratio of 1.4:1, and constitutes 14% of all leukemias (Lee, 2000).

CML progresses through three distinct phases: the stable or chronic phase, the accelerated phase and blast crisis. The chronic phase is characterised by massive expansion of myeloid cells, which maintain normal maturation. Patients with chronic phase CML have effective immune systems and generally feel well for prolonged periods. Common symptoms are often mild and include fatigue, weight loss, body aches and discomfort caused by splenomegaly (Lee, 2000). In the later phases, leukemic cells lose their capacity to terminally differentiate; the result is an acute leukemia, which is highly refractory to therapy.

1.3.1 BCR/ABL

The cytogenetic hallmark of all phases of CML and a subset of cases of acute lymphoblastic leukemia (ALL) is the Philadelphia (Ph) chromosome, designated after the city where the abnormality was first recorded. The discovery of the Philadelphia chromosome in 1960, as the first consistent chromosomal abnormality associated with a specific type of leukemia, was a breakthrough in cancer biology.

The Ph chromosome is a shortened chromosome 22 that results from a reciprocal translocation between the long arms of chromosomes 9 and 22. The molecular consequence of this translocation is the fusion of the c-*Abl* oncogene from chromosome 9 with sequences from chromosome 22, the breakpoint cluster region (*bcr*), giving rise to a fused *bcr/abl* gene. Depending on the site of the breakpoint in *bcr*, various different fusion proteins are produced: p185 (185 kDa), p210 (210 kDa), or rarely p230. All three BCR/ABL fusion proteins exhibit deregulated tyrosine kinase activity compared with the native ABL protein and

are associated with malignant transformation. Tyrosine kinases are enzymes that transfer phosphate from ATP to tyrosine residues on substrate proteins that in turn regulate cellular processes, such as proliferation, differentiation, and survival. Therefore it is not surprising that deregulated tyrosine kinase activity has a central role in malignant transformation. The p210 protein is seen in 95% of patients with CML and up to 20% of adult patients with *de novo* ALL (acute lymphocytic leukemia); the p185 form is seen in approximately 10% of patients with ALL and in the majority of pediatric patients with Ph+ ALL (5% of all pediatric ALL cases), p230BCR/ABL is associated with the very rare Philadelphia chromosome (Ph)-positive chronic neutrophilic leukemia (Mauro and Druker, 2001).

The role of BCR/ABL in inducing CML has been shown using a number of different systems: transgenic murine model systems have established its ability to induce leukemia and retroviral transduction of BCR/ABL into murine bone marrow has reproduced a CML-like myeloproliferative disorder (Daley et *al.* 1990).

1.3.1.1 c-*Abl*

The c-*Abl* gene is a member of the family of non-receptor tyrosine kinases consisting of the normal cellular proteins c-Abl and Arg. c-Abl is the cellular homologue of the transforming protein found in the Abelson murine leukemia virus (v-Abl).

The Abelson murine leukemia virus was first isolated from a mouse that developed a pre-B lymphoma following introduction of Moloney murine leukemia virus (Abelson and Rabstein, 1970). The viral genome was found to contain viral *gag* sequences and a novel gene, later identified as the c-terminus of murine c-Abl.

c-*Abl* comprises two alternative first exons, 1a and 1b, and common exons 2-11 (Holyoake, 2001). Human ABL is a ubiquitously expressed 145 kDa protein with 2 isoforms arising from alternative splicing of the first exon and contains several function domains: SH3, SH2, SH1 domains in the N-terminal region, a nuclear localisation signal (NLS), a proline-rich region capable of associating with SH3-containing proteins, a sequence-independent DNA-binding domain (DB), and an actin-binding domain (AB) (Figure 1.6) (Chung and Wong, 1995; Zou and Calame, 1999). c-Abl appears to have a major role in regulating cell cycle progression. In the nucleus in quiescent cells, the Abl activity is inhibited by binding a complex involving the retinoblastoma (Rb) protein. However, as cells progress from the G1 phase to the S phase, pRB is phosphorylated, dissociates from Abl, which then becomes activated and is phosphorylated by CDC2 kinases, with subsequent effects on transcription and progression to S phase (Wang, 2000). c-Abl activity is positively regulated by DNA damage signals. Cells lacking c-Abl can activate cell cycle checkpoints and DNA repair, but show defects in apoptosis, correlated with a defect in the induction and activation of p73. c-*Abl* knockout mice have perinatal mortality and display facial abnormalities. Also, the spleen is abnormal, and development of T cell, as well as B cell progenitors, in the bone marrow is reduced (Tybulewicz *et al.*, 1991). Thus, c-Abl is a potent transforming oncoprotein, and understanding its immediate substrates and final targets will help in understanding the processes required for malignant transformation.

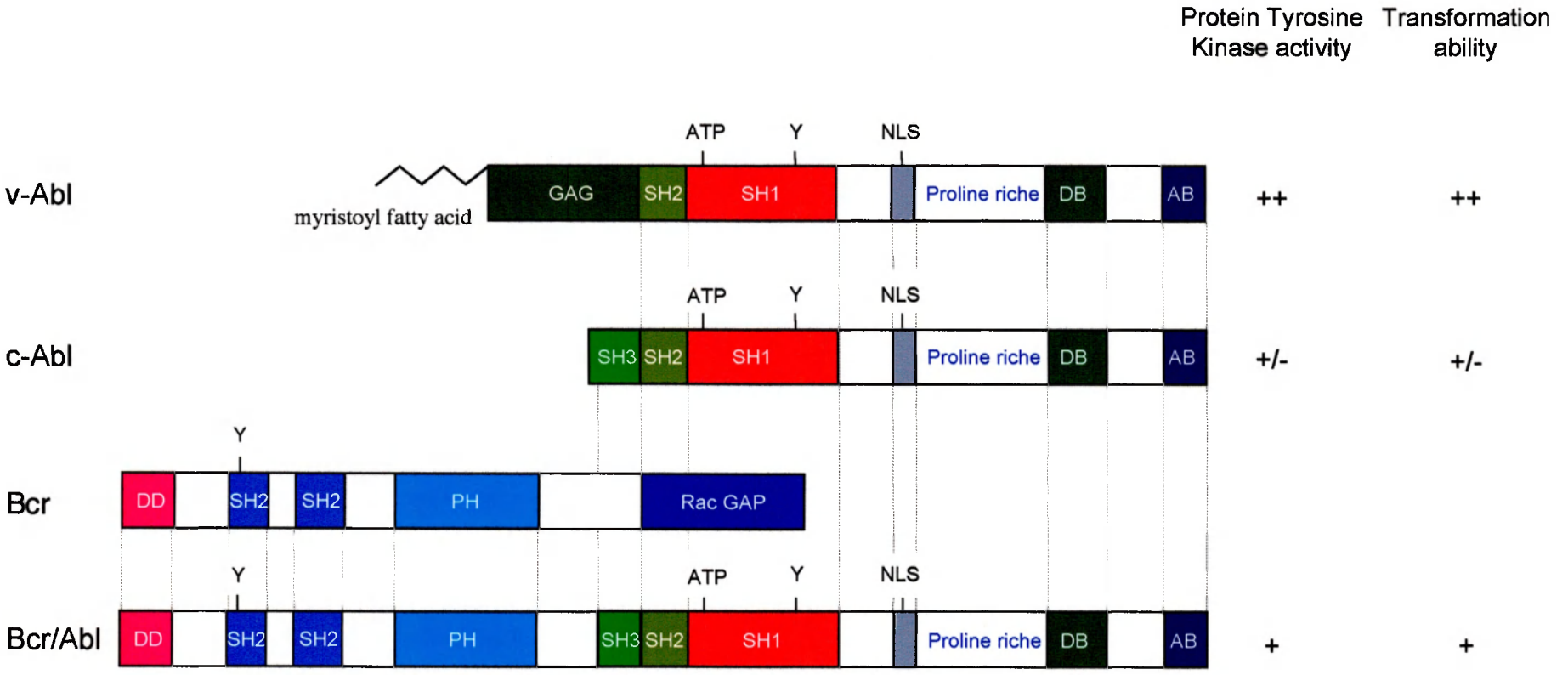

Figure 1.6. Functional motifs of Abl family protein tyrosine kinases.

SH, Src homology domains; NLS, nuclear localization signal; DB, DNA-binding domain; AB, actin-binding domain; ATP, the ATP-binding site in the kinase domain; Y, the main site of autophosphorylation; GAG, retroviral Gag domain; BCR, breakpoint cluster sequence.

1.3.1.2 BCR gene and protein

The break point cluster region (BCR) gene on the chromosome 22 comprises 23 exons in a region of 135 kb. Gene expression results in transcription into two mRNAs, of 4.5 kb and 7 kb, both of which are translated into a 160 kDa protein. BCR is ubiquitously expressed, with the highest levels in brain and haematopoietic tissues, and it is localised in the cytoplasm. The BCR protein has multiple functional domains, the most critical of which is the dimerisation domain (DD) at the N-terminus, responsible for the homotetramerisation of the BCR/ABL molecule and necessary for its transforming potential (Tauchi *et al.*, 1997). This domain is followed by a unique Serine-Threonine kinase domain, including at least three SH2 binding sites and particularly a tyrosine (TYR 177), the autophosphorylation of which is necessary for binding to GRB2 and activation of the Ras pathway and beta-isoform of 14-3-3 proteins. The centre of the molecule contains a pleckstrin-homology (PH) domain that stimulates the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on Rho guanidine exchange factors, which in turn may activate transcription factors, for instance NF-κB. The C-terminus domain functions as a GTPase activating protein for p21rac, a small GTPase of the Ras superfamily that regulates actin polymerisation and the activity of an NADPH oxidase in phagocytic cells (Deininger *et al.*, 2000; Holyoake, 2001).

1.3.1.3 Signalling pathways activated by oncogenic forms of Abl tyrosine Kinase

The increased tyrosine kinase activities of v-Abl and BCR/ABL, compared with the cellular c-Abl, correlate with their transforming activities. The molecular mechanisms by which these activated tyrosine kinases cause malignant transformation have remained obscure until the last few years, when there have been reports that multiple signalling pathways are activated by v-Abl and/or BCR/ABL (Guo *et al.*, 1998).

v-Abl

The v-*Abl* oncogene in the Abelson murine leukemia virus encodes a fusion protein in which a portion of retroviral Gag protein replaces the SH3 domain of c-Abl (Figure 1.6) (Abelson and Rabstein, 1970). Removal of the SH3 domain constitutively activates the tyrosine kinase, and a myristoylation site in the Gag moiety confers localization to the inner plasma membrane; both modifications are important in the transforming activity of v-Abl (Prywes *et al.*, 1983). Infection of neonatal mice by the Abelson murine leukemia virus results in rapid, 100% fatality because of pro/preB cell tumors. Although *in vitro* v-Abl transforms pro/preB cells as well as myeloid cells and a subset of 3T3 fibroblasts (Renshaw *et al.*, 1992), *in vivo* it only transforms pro or preB cells, that is the early B-lineage cells that have partially or completely rearranged their heavy chain genes (Rosenberg, 1994). This striking pro/preB cell tropism for transformation, in the absence of any evidence of pro/preB-specific viral infection, is likely to reveal regulatory paths that are unique to the early B-lymphocyte lineage.

Data from a variety of experiments show that signalling through the GTP-binding protein Ras is essential for transformation by v-Abl. Inhibition of p21Ras activity by antisense oligonucleotides, microinjection of a blocking monoclonal antibody to p21Ras or expression of the catalytic domain of Ras GAP all block transformation by v-Abl (Sawyers *et al.*, 1995; Skorski *et al.*, 1994; Smith *et al.*, 1986).

v-Abl has been shown to activate phospholipase C-mediated breakdown of phosphatidylcholine, generating diacylglycerol, which then activates the Protein Kinase C (PKC) (Owen *et al.*, 1993). Subsequent studies showed that the v-Abl/PKC pathway causes an increase in *bcl*-Xl mRNA levels (Chen *et al.*, 1997).

Evidence is accumulating to support a role for JAK binding and STAT in v-Abl transformation. JAK-1 and JAK3 associate directly with v-Abl (Danial *et al.*, 1998). In v-Abl-transformed cells, STAT1, STAT5 and STAT6 have been shown to be constitutively activated (Danial *et al.*, 1995). Deletion of 200 amino acids in the DNA-binding portion of the v-Abl C-terminus that are required for association with JAK1 results in a mutant v-Abl which cannot provide cytokine-independent survival of BaF3 pro-B cells.

BCR/ABL

All BCR/ABL proteins have tyrosine kinase activities intermediate between the weaker c-Abl and the stronger v-Abl (Clark *et al.*, 1987). *In vitro* BCR/ABL expression confers growth factor independence, but is fully transforming only for certain cells (Raitano *et al.*, 1997).

BCR/ABLp210 contains the first 927 amino acids of BCR and its transforming ability is provided by multiple functional domains, as shown by mutating critical residues in Src homology 2 domain (R552L), the autophosphorylation site in the

kinase domain (Y793F), or the GRB2 binding site (Y177). The combination of all three mutations blocked the antiapoptotic activities of BCR/ABL in haematopoietic cells (Cortez *et al.*, 1995), while single mutations just reduced transformation by BCR/ABL (McWhirter and Wang, 1993; Pendergast *et al.*, 1993).

The first 63 amino acids of the BCR part in the fusion protein forms an α-helical coiled-coil structure, which leads to the oligomerisation of BCR/ABL molecules, autophosphorylation and activation of the catalytic activity (McWhirter *et al.*, 1993). This domain is also involved in the localisation of BCR/ABL by its ability to interact with F-actin. Whereas the c-Abl protein shuttles between the nucleus and the cytoplasm, due to the presence of nuclear localisation and export signals (NLS and NES) within its C-terminal region, the BCR/ABL fusion proteins are exclusively cytoplasmic. This cytoplasmic localisation of BCR/ABL seems to be essential for its biologic activities because BCR/ABL entrapment in the nucleus by leptomycin B induces apoptosis with its tyrosine kinase activities (Vigneri and Wang, 2001).

Basic mechanisms thought to be relevant in the pathogenesis of Ph-positive leukemias are activation of mitogenic signalling, inhibition of apoptosis and altered adhesion to stroma. BCR/ABL leads to tyrosine phosphorylation of multiple proteins and, therefore, simultaneous activation of multiple signalling pathways, but little is known about their downstream targets. A host of substrates can be tyrosine phosphorylated by BCR/ABL, summarised in Table 2, below. Most important, because of autophosphorylation, there is a marked increase of phosphotyrosine on BCR/ABL itself, which creates binding sites for the SH2 domains of other proteins (Carlesso *et al.*, 1996; Tauchi *et al.*, 1997).

Protein	**Function**
Crkl	Adapter
Crk	Adapter
Shc	Adapter
Talin	Cytoskeleton/cell membrane
Paxillin	Cytoskeleton/cell membrane
Fak	Cytoskeleton/cell membrane
Fes	Myeloid differentiation
Ras-GAP	Ras-GTPase
PLCγ	Phospholipase
PI 3-Kinase	Serine Kinase
Grb2	Adapter
Vav	Haematopoieitc differentiation
Bap-1	14-3-3 protein

Table 2. Substrates of BCR/ABL.

Generally, substrates of BCR/ABL can be grouped, according to their physiological role, into adapter molecules, proteins associated with the organisation of the cytoskeleton and the cell membrane, and proteins with catalytic function. Some phenotypic correlations can be made with the molecular abnormalities:

1. Constitutive activation of the Ras pathway (via TYR177 of the BCR) mimicking the growth-factor-stimulation of cells, leads to a proliferative behavior. Expression of dominant-negative Ras in BCR/ABL expressing K562 cells resulted in apoptotic cell death (Sakai *et al.*, 1994). The activation of Ras is dependent on SOS and Ras-GAP and, in CML cells, evidence has been found for constitutive Ras activation and reduced Ras-GAP activity (Skorski *et al.*, 1994). The mechanism of Ras activation appears to be mediated through the adapter proteins GRB2 and Shc, which have been demonstrated to be

associated with BCR/ABL (Skorski *et al.*, 1995b). The constitutive activation of Ras in BCR/ABL transformed cells is likely to lead to an anti-apoptotic pathway required, but insufficient, for transformation (Cortez *et al.*, 1995).

2. Activation of PI 3-K/AKT as well as the JAK/STAT pathway is most likely responsible for the anti-apoptotic potential. There is increasing evidence that PI 3-K is important in transformation by BCR/ABL. PI 3-K activity has been shown to be regulated by BCR/ABL and required for growth of CML cells, using antisense oligodeoxynucleotides to reduce PI 3-K expression. Further, wortmannin, a specific inhibitor of the p110 subunit of PI 3-K, was shown to inhibit proliferation of BCR/ABL transformed cells, but not normal haematopoietic cells (Calabretta and Skorski, 1996; Skorski *et al.*, 1995a). Constitutive phosphorylation of STAT1 and STAT5 was observed in both cells transformed by BCR/ABL and in Ph+ human leukemia cell lines. Using thermosensitive mutants this phosphorylation was shown to be directly due to the tyrosine kinase activity of BCR/ABL (Ilaria and Van Etten, 1996; Nieborowska-Skorska *et al.*, 1999). In contrast, JAK kinases are not consistently activated in BCR/ABL positive cells and activation of STAT5 by BCR/ABL is not blocked by dominant-negative JAK2 mutants. (Carlesso *et al.*, 1996; Chai *et al.*, 1997; Ilaria and Van Etten, 1996). These data suggest that BCR/ABL might activate STATs by a JAK-independent pathway.

   Studies have indicated that the members of the anti-apoptotic pro-apoptotic bcl-2 family BAD, Bcl-2 and Bcl-Xl are downstream targets of STATs. BAD, *Bcl*-2 and *Bcl*-XL expression restored the leukemogenic potential of a BCR/ABL mutant defective in transformation in 32Dcl3 cells (Cirinna *et al.*, 2000) and after ectopic expression of Bcl-2, 32Dcl3 cells proliferated in the absence of IL-3. All the three proteins have been shown to be regulated by

STATs (Sanchez-Garcia and Grutz, 1995). BCR/ABL was shown to increase the transcription of *Bcl*-XL promotor and the protein levels through the STATs. Moreover, this induction was blocked by the expression of a dominant negative STAT5 mutant. (Gesbert and Griffin, 2000).

3. Activation of focal adhesion molecules (FAK/paxillin) via CRK-L, as well as an abnormal response to SDF-1, leads to adhesive and migratory abnormalities of leukemic cells. Studies have shown that CML cells have altered adhesion to marrow stromal cells and some extracellular matrix proteins, notably fibronectin (Gordon *et al.*, 1987).

1.4 Aims of this thesis

It is believed that BCR-ABL activates similar intracellular signalling pathways to IL-3 to promote proliferation and survival in cytokine-dependent haematopoietic cells. Therefore, BCR/ABL might regulate the same set of genes that are controlled by IL-3. The identification of the abnormalities that result in cancerous growth can lead to the understanding of the mechanisms that control normal growth and development.

Major cellular effects of BCR/ABL are related to increased mitogenic activity and reduced sensitivity to apoptosis. BCR/ABL reduces the growth factor requirements of primary haematopoietic stem cells, converts IL-3-dependent murine haematopoietic cells lines to growth factor independence and it is mitogenic in fibroblasts. BCR/ABL is likely to bypass normal controls on the proliferation of haematopoietic cells by altering the expression of cell cycle regulatory proteins through aberrant activation of upstream signalling pathways,

thus one purpose of this investigation was to identify the downstream targets and their physiological roles.

Although several signalling pathways have previously been reported to play essential roles in BCR/ABL transformation, the exact mechanism by which the proliferative signals transduce from BCR/ABL and IL-3 to the pRB pathway is not yet well defined. The second aim of this work was to determine the signalling pathways by which BCR/ABL and IL-3 mediate proliferation and survival in haematopoietic cells.

Chapter 2. MATERIAL AND METHODS

2.1 Reagents and buffers

6x Agarose Gel Loading Buffer

0.25% (w/v)	Bromophenol blue
0.25% (w/v)	Xylene cyanol
30% (v/v)	Glycerol

Ampicillin (Sigma)

A stock solution of 100mg/ml was made in ddH_2O and stored at –20°C. The final concentration used in 2x TY medium was 100µg/ml.

1x Bacteria transforming buffer

100mM	$CaCl_2$
70mM	$MnCl_2$
40mM	Na acetate

pH adjusted to 5.5

Blotto

10% (w/v)	fat-free dried milk powder reconstituted in PBST

Doxycyclin (Sigma, UK)

Doxycyclin was added to the media at the appropriate concentration from a 1mg/ml fresh solution in ddH_2O.

1x Gilbert and Church hybridisation buffer

7% (w/v)	BSA
1mM	SDS
200mM	$NaPO_4$ pH 7.2
45% (v/v)	Formamide

LY294002 (Calbiochem, UK)

A stock solution of 50mM was made up in DMSO and stored at –20°C. The final concentration used was 50µM.

10x Morpholinopropanesulphonic acid (MOPS) buffer

200mM	MOPS pH 7.0
50mM	Sodium acetate
10mM	EDTA

Phosphate-buffered saline (PBS)

12.5mM	NaCl
2mM	Na_2HPO_4
1mM	$NaH_2PO_4,2H_2O$

pH adjusted to 7.2

Phosphate-buffered saline – tween (PBST)

0.05% (v/v) Tween20 (Polyoxyethylene-sorbitan monolaurate) in PBS

Propidium Iodide staining buffer

0.1% (v/v)	Triton X-100
0.1% (w/v)	$Na_3C_6H_5O_7.2H_2O$ (sodium citrate)

RIPA buffer

1% (v/v)	NP-40
0.5% (w/v)	Sodium deoxycholate
0.1% (w/v)	SDS
2 tablets	Complete™ protease inhibitor cocktail (Boehringer Mannheim) per 10ml

Made up in PBS

RNA loading buffer

50% (v/v)	Glycerol
1mM	EDTA
0.4% (w/v)	Bromophenol blue
0.4% (w/v)	Xylene cyanol

2x SDS-PAGE loading buffer

4% (w/v)	SDS
62.5mM	Tris.HCl pH 6.8
1% (v/v)	Glycerol
0.01% (w/v)	Bromophenol blue
10% (v/v)	2-mercaptoethanol

10x SDS-PAGE running buffer

250mM	Tris.base
2M	Glycine
1% (w/v)	SDS

10x SDS-PAGE transfer buffer

250mM	Tris.base
2M	Glycine

20x Sodium citrate-sodium chloride (SSC)

3M	NaCl
300mM	Sodium citrate
pH adjusted to 7.0	

STI571 (Novartis)

A stock solution of 10mM was made in distilled water and stored at 4°C. STI571 was a gift from E. Buchdunger.

1x Tail lysis buffer

100mM	Tris.HCl (pH 8.8)
0.02% (w/v)	SDS
5mM	EDTA (pH 8.0)
200mM	NaCl

50x Tris-sodium acetate-EDTA (TAE) buffer

2M	Tris base
0.05M	EDTA
57ml	glacial acetic acid

Tris-EDTA (TE) buffer

10mM	Tris.HCl pH 7.6
1mM	EDTA pH 8.0

2x TY broth

1.6% (w/v)	Bacto-tryptone
1% (w/v)	Yeast extract
85mM	NaCl
pH adjusted to 7.4	

2.2 Enzyme Unit Definitions

Calf intestinal alkaline phosphatase (CIAP)

One unit catalyses the hydrolysis of 1 μmol of 4-nitrophenyl phosphate per minute at 37°C in 1M diethanolamine, 10.9mM paranitrophenyl phosphate, 0.5mM $MgCl_2$ (pH 9.8).

Restriction endonucleases

One unit digests 1μg of phage λ DNA to completion in 60 minutes at 37°C in a 50μl reaction volume.

Taq DNA polymerase

One unit incorporates 10nmol of total deoxyribonucleotides (dNTPs) into acid insoluble DNA in 30 minutes at 74°C under standard DNA polymerase assay conditions.

T4 DNA ligase

One Weiss unit of enzyme catalyzes the exchange 1nmol of ^{32}P from pyrophosphate into Norit-absorbable substance in 20 minutes at 37° C. 0.01 Weiss unit of ligase is equivalent to 1 ligation unit which catalyses greater than 95% ligation of 1μg lambda/HindIII fragments in 20 minutes at 16° C.

2.3 Antibodies

Primary Antibodies

Antibody	Clone	Specie	Dilution	Source
$p16^{INK4a}$	m-156	Rabbit polyclonal	1:2000	Santa Cruz Biotechnology, Inc.
$p21^{Cip1}$	F-5	Mouse monoclonal	1:2000	Santa Cruz Biotechnology, Inc.
$p27^{Kip1}$	C-19	Rabbit polyclonal	1:2000	Santa Cruz Biotechnology, Inc.
pRB	-	Mouse monoclonal	1:1000	PharMingen
P-pRB	Thr821	Rabbit polyclonal	1:1000	Biosource, International
P-pRB	Ser807/811	Rabbit polyclonal	1:500	New England Biolabs
p107	C-18	Rabbit polyclonal	1:2000	Santa Cruz Biotechnology, Inc.
p130	C-20	Rabbit polyclonal	1:2000	Santa Cruz Biotechnology, Inc.
Cyclin A	C-19	Rabbit polyclonal	1:2000	Santa Cruz Biotechnology, Inc.
Cyclin D2	M-20	Rabbit polyclonal	1:2000	Santa Cruz Biotechnology, Inc.
Cyclin D3	18B6-10	Rat monoclonal	1:2000	Santa Cruz Biotechnology, Inc.
Cyclin E	M-20	Rabbit polyclonal	1:2000	Santa Cruz Biotechnology, Inc.
CDK2	M-2	Goat polyclonal	1:2000	Santa Cruz Biotechnology, Inc.
CDK4	C-22	Goat polyclonal	1:2000	Santa Cruz Biotechnology, Inc.
CDK6	C-21	Rabbit polyclonal	1:2000	Santa Cruz Biotechnology, Inc.
ABL	Ab-3	Mouse monoclonal	1:1000	Calbiochem
AKT	-	Rabbit polyclonal	1:1000	New England Biolabs
P-AKT	Thr 308	Rabbit polyclonal	1:1000	New England Biolabs

Secondary Antibodies

Antibody	Specie	Dilution	Source
Anti-mouse IgG	Goat	1:2000	Dako
Anti-mouse IgG	Goat	1:2000	Dako
Anti-mouse IgG	Mouse	1:2000	Southern Biotechnology associates, Inc

2.4 Western Immunoblotting

Protein isolation

For total cellular lysates, cell pellets were resuspended in 2 times packed cell volume of RIPA buffer and incubated on ice for 10 minutes. Cellular debris was spun down at 14,000 rpm for 10 minutes in a microfuge and the lysate was transferred to a fresh tube. The protein concentration was assayed using the Bio-Rad DC kit protein (Bio-Rad, USA).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Fifty μg of whole cellular protein extracts were mixed with one volume of 2x SDS-PAGE loading buffer and boiled for 5 minutes prior to loading onto an SDS-polyacrylamide gel and separation at 40V overnight.

The gels consisted of a 4% stacking gel above a resolving gel.

	Resolving gel		Stacking gel
	5%	12%	4%
30% (v/v) Acrylamide	5.5ml	13.3ml	3.3ml
1.5 M Tris pH 8.8	8.3ml	8.3ml	-
0.5 M Tris pH 6.8	-	-	6.3ml
10% SDS	0.3ml	0.3ml	0.25ml
H_2O	19.0ml	11.2ml	15ml
25% (w/v) APS	0.05ml	0.05ml	0.03ml
TEMED	0.05ml	0.05ml	0.03ml

Rainbow™ marker High molecular weight range (14.300 – 220,000 Da) (Amersham LifeScience) was used as a size standard.

Transfer to Nitrocellulose Membrane

The resolved proteins were electrophoretically transferred on to a nitrocellulose membrane (Schleier and Schuell, Dassel, Germany). The transfer was performed

in SDS-PAGE transfer buffer in the presence of 23% (v/v) ethanol at 140V for a minimum of 5 hours.

Immuno-detection

The membrane was blocked with blotto for 20 minutes, followed by overnight incubation with the primary antibody at 4°C. The membrane was then washed with PBST and incubated with the appropriate secondary horseradish peroxidase-conjugated antibody for 20 minutes at room temperature. After extensive washing with PBST the proteins were visualised using enhanced chemiluminescence fluorography (ECL, Amersham International, UK) and exposed to Hyperfilm™ MP (Amersham International, UK).

2.5 DNA preparation and Manipulation

Preparation of heat-shock competent bacteria

500ml of 2x TY was inoculated with 1ml of a fresh overnight culture of HB101 bacteria strain and incubated at 37°C until the culture reached an optical density of 0.5 at 600nm. The cells were then chilled on ice for 2 hours, pelleted by centrifugation (2000g for 10 minutes) and resuspended in 250 ml of 1x bacteria transformation buffer. After incubation on ice for 40 minutes, the cells were pelleted as before and resuspended in 25ml of the transformation buffer containing 15% glycerol. This suspension was stored at -70°C in 200µl aliquots.

Transformation of Competent Bacteria with Plasmid DNA

One microlitre of plasmid DNA was mixed with 40µl of heat-shock competent bacteria and incubated on ice for 5 minutes. The bacteria were then heat-shocked for 1 minute at 42°C and placed on ice for another 5 minutes. The bacteria were

plated onto TY plates supplemented with ampicillin (75μg/ml), and incubated overnight at 37°C.

Small Scale Preparation of Plasmid DNA from Bacteria (Miniprep)

Three ml of 2x TY were inoculated with a single colony of bacteria and grown at 37°C for 6 hours. A 1ml sample was centrifuged at 14,000rpm in a microfuge for 1 minute and the pellet resuspended in 300μl of STE buffer. The proteins were removed by phenol:chloroform extraction and the DNA precipitated by the addition of 600μl of 100% ethanol. After centrifugation at 14,000rpm in a microfuge for 5 minutes the pellet was air-dried and resuspended in 20μl H_2O. Ten μl of the sample was digested and analysed on an agarose gel.

Large Scale Plasmid Preparation (Maxiprep)

500ml of 2x TY was inoculated with 5ml of exponentially growing culture and incubated overnight at 37°C. The cells were pelleted by centrifugation at 2000g for 5 minutes at 4^0C. The plasmid DNA was purified using a Qiagen Plasmid Mega Kit following the manufacturer's instructions. To eliminate any protein contamination, the DNA was phenol:choloroform extracted before ethanol precipitation.

Quantification of DNA

The concentration of DNA was determined by measuring the absorbance at 260nm where the value of 1.0 corresponded to 50μg/ml of DNA. The quality of purified DNA was also estimated by measuring the ratio of OD 260 / OD 280 where a value of 1.8-2.0 indicated purified DNA.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed in 1-1.6% (w/v) agarose (Ultra-pure agarose, Gibco BRL) in TAE containing 1μg/ml ethidium bromide at 5-8V/cm. DNA samples were prepared in 1x sample buffer.

Subcloning of DNA Fragments

DNA digestion

Plasmids were digested using 2 units of restriction enzyme per microgram of DNA in the appropriate 1x restriction endonuclease buffer (New England Biolabs, UK). Reactions were incubated for a minimum period of 2 hours at the manufacturer's recommended temperature. After electrophoresis, the fragments of interest were excised and purified from the agarose gel.

Dephosphorylation of vector DNA

To prevent re-ligation of linearised vectors, the 5' phosphate groups were removed. 5U of CIAP was added to the restriction reaction and incubated for 30 minutes at 37^{0}C. The phosphatase was inactivated by incubation at 75^{0}C for 15 minutes.

DNA ligation

Vectors and DNA fragments were quantified by agarose gel electrophoresis and the ligation was set up such that the ratio of the insert:vector DNA was 3:1. A typical reaction consisted of the following:

150ng	insert DNA
50ng	vector DNA
2μl	10x T4 DNA ligase buffer
1μl	T4 DNA ligase (400,000U/ml)
ddH_2O to 20μl	

The reactions were incubated overnight at room temperature. Afterwards, competent bacteria were transformed with the ligated DNA reaction mix and grown in TY media.

Phenol/Chloroform Extraction and ethanol Precipitation

DNA was mixed by vortexing with an equal volume of Phenol/Chloroform/Isoamyl Alcohol 25:24:1 (v/v) (SIGMA). The phases were separated by centrifugation at 16,000g for 5 minutes at room temperature. The aqueous phase was then carefully removed and transferred to a new tube and the DNA was precipitated by incubation on dry ice with 1/10 volume of 3M NaCl and 2 volumes of ethanol. The DNA was collected by centrifugation at 16,000g for 10 minutes at 4^0C, The pellets were washed once in 70%(v/v) ethanol, air-dried and resuspended in TE.

Gel Purification of DNA Fragments

DNA fragments were separated by electrophoresis and the DNA band required was excised using a razor blade. The fragments were purified using the Geneclean II kit (bio101) according to the manufacturers instructions.

2.6 Northern blot

RNA extraction

Total RNA was extracted following the protocol provided with the RNeasy kit from Qiagen (Qiagen Ltd, UK). The concentrations were determined by measuring the optical density at 260nm.

RNA electrophoresis and Northern blotting

Twenty μg of total RNA were mixed with 15μl of a solution of 65% (v/v) formamide, 22% (v/v) formaldehyde and 13% (v/v) 10x MOPS buffer. The samples were then heated at 70°C for 5 minutes, after which 2μl of RNA loading buffer was added. The samples were loaded onto a 1.4% (w/v) agarose gel containing 1x MOPS buffer, 10% (v/v) formaldehyde and 1μg/ml ethidium bromide. The gel was run overnight at 30V in 1x MOPS buffer. The RNA was transferred onto a Hybond™ –XL nylon membrane using capillary transfer for 30 hours. It was then crosslinked to the membrane by ultraviolet exposure (Spectrotronics Corp.) at 120mJ/cm^2.

Preparation of Radiolabelled DNA probes

Fifty ng of DNA were denatured on a heating block at 100°C for 10 minutes and quickly chilled on ice for 5 minutes. The denatured DNA was added together with 5μl of Redivue [α-^{32} P] dCTP (10mCi/ml) to a Rediprime random primer labelling tube (Amersham LifeScience) and incubated at 37°C for 30 minutes. Free deoxynucleotides were removed by passing the probe over a Nick™ G-50 Sephadex column. The probe was added to the pre-equilibrated column and 400μl of TE was used to wash the probe into the column. The probe was eluted and collected adding another 400μl of TE to the column.

Hybridisation

The Northern blot membrane was prehybridised at 65°C for 30 minutes in hybridisation buffer. Then hybridised overnight at 42°C with 200μl of denatured labelled probe. The Northern blot was washed several times in 2x SSC, 0.2% (w/v) SDS, followed by washes in 1x SSC, 0.1% (w/v) SDS till the counting <10 Bcq. The filter was exposed to auroradiograph film.

2.7 Mammalian Cell Manipulation

Cell lines used

NAME	SOURCE	REFERENCE
BaF3	Dr. M. Gordon	(Ormerod et al., 1992)
BaF3-p210	Dr. M. Gordon	(Daley et al., 1991)
TonB210.1	Dr. G. Daley	(K.M. Clucher et al., 1998)

All cells were maintained in RPMI-1640 (Gibco BRL, UK), supplemented with 10% foetal calf serum (FCS), 2mM L-Glutamine (Gibco, BRL, UK), 100U/ml Penicillin-Streptomycin (Gibco, BRL, UK) and 10% (v/v) WEHI-3B conditioned medium as a source of IL-3. Cells were maintained at 37°C in a humidified Heraeus incubator at a CO_2 concentration of 10%.

Cryopreservation of cells

A 25 ml sample of cell culture at a density of $2x10^6$ cells/ml was centrifuged at 490g for 5 minutes at 4°C. The supernatant was removed and the cell pellet was resuspended in 1ml freezing medium (DMSO 10% and FCS 90%). Cell aliquots of 1ml were transferred to screw cap freezing tubes (Nalgene), placed in a Nalgene cryo freezing box containing propan-2-ol, and left at -80°C for one day, cooling the samples at a rate of approximately 1°C per minute. Thereafter the ampoules were stored in liquid nitrogen.

Counting of cells.

Ten μl of cell suspension was added to 10μl of trypan blue (Sigma, UK), mixed together and then introduced to the haemocytometer chamber (Weber, UK). Viable cells were identified as those cells excluding the trypan blue dye.

Transfection of Mammalian Cells

Transient co-transfection of Baf3 cells

Cells were split 1:4 24 hours prior to transfection, resulting in a cell density of approximately $1.5x10^6$ cells/ml. Per transfection, $1x10^7$ cells were spun at 490g at 4°C for 3 minutes and resuspended in 150µl RPMI medium. Cells were transfected by standard electroporation (0.28 kV and 960µF). After electroporation the cells were left to recover in a 37°C incubator for 20 minutes and then transferred to 10ml of medium and returned to the incubator. 3 hours after electroporation, dead cells were removed by separating through a Ficoll gradient (1000g for 30 minutes at 4°C). The DNA of interest was cotransfected with spectrin-GFP as a marker for transfected cells. After 24 hours cells were analysed by FACS unless stated otherwise.

Stable transfection

Constructs were electroporated into BaF3 cells together with pBABE containing a puromycin selection marker and selected for 3 days with increasing concentrations of puromycin (0.5-2.0µg/ml)

Transduction of TAT Fusion Proteins

The TAT fusion proteins TAT-$p27^{Kip1}$WT, TAT$p27^{Kip1}$KK and TAT-eGFP were obtained from Dr. S. Thomas, Department of Haematological Medicine, Guy's, King's. St. Thomas' School of Medicine, London. Cell lines were transduced by adding the appropiate TAT-protein to the medium at a final concentration of 100-500nM. After 24h and 48h cells were analysed by FACS.

FACS

Propidium Iodide Staining

The cells were harvested by centrifugation at 1,300rpm (Sorval RC3C) for 3 minutes. After washing once with PBS, the cells were fixed in 90% (v/v) ethanol, 10% (v/v) PBS overnight at 4°C. The cells were washed once with PBS and incubated in PI staining buffer containing 10μg/ml Rnase (SIGMA, UK) at 37°C for 30 minutes, followed by a second incubation of 30 minutes at 37°C in the presence of PI at 20μg/ml.

Annexin V

Cells were stained using the Annexin V kit (R&D, Systems Europe Ltd, Oxon, UK) according to the manufacturer's instructions.

FACS analysis

Cell profile was analysed using a Becton Dickinson FACSort analyser, combined with the CELLQuest software. Control stained cells were used to set up the amplifier gains and detector voltages for the detector parameters. Using a FSC-H vs. SSC-H dot plot cells were selected in a gate in order to avoid clumps. 10,000 gated events were collected and shown as a histogram of counts vs. FL2-A and the %G0+G1, %S and %G2/M phases were measured.

PCR

Mouse tails were digested overnight at 55°C in 500μl of 1x Tail lysis buffer supplemented supplemented with proteinase K at a final concentration of 0.5 mg/ml. After centrifugation, the supernatant was transferred to a new eppendorf tube and the DNA precipitated using 0.7 volumes of iso-propanol. The DNA was subsequently amplified by PCR using specific primers for the vector disrupting

the gene to detect the mutant and primers for the undisrupted gene to detect the wild type. The hybridisation temperature varied depending on the primers used and the quality of the DNA extracted.

A typical PCR sample contained the following:

100µl 10X Taq polymerase buffer (Promega)
5µl 2mM each dATP,dCTP,dGTP,dTTP
100µl 25mM $MgCl_2$
10µl Taq polymerase (5000U/ml)
5µl each primer (50pmol each)
5ng template DNA

Reactions were carried out in 0.5ml centrifuge tubes and overlaid with a single drop of mineral oil (Sigma, USA) to prevent evaporation.

The thermocycle programme was usually set for 39 amplification rounds at:

Denaturing: 93^0C 1 minute

Annealing: 53^0C 1 minute

Elongation: 72^0C 1 minute

The PCR programme was followed by a 7 minutes hybridisation extension at 72^0C.

Chapter 3. Regulation of cell cycle regulatory proteins by BCR/ABL and IL-3 in haematopoietic cells.

3.1 Introduction

IL-3 was initially identified and cloned on the basis of its ability to induce proliferation of marrow cells in the absence of growth factors. In 1968, Noel Warner induced the development of a large series of primary murine tumours, mostly plasmacytomas, by the injection of mineral oil or pristane into responsive BALB/c mice (Warner *et al.*, 1969). One of these primary animals (WEHI-3) appeared highly unusual in that the enlarged lymph nodes were greenish in colour. Analysis showed that this tumour was in fact a rare myelomonocytic leukemia and transplantation studies led to the derivation of four very different sublines of transplantable tumours (WEHI-3AD). Interestingly, cocultures of the original WEHI-3B tumour cells with normal marrow cells led to colony formation demonstrable as originating from the cultured normal cells, suggesting that the cell line had an unusual capacity to produce CSF. More remarkable was the curious appearance of the small numbers of developing colonies. These were composed of uniformly dispersed cells in globular clouds and of small clusters of giant cells. In 1973, pokeweed mitogen-stimulated spleen-conditioned medium (SCM) proved able to stimulate the formation of the same curious dispersed colonies as had developed in the earlier cocultures of marrow cells with WEHI-3B cells (Parker *et al.*, 1973), and later on, its ability to stimulate multipotential and erythroid colony formation in cultures of mouse foetal liver cells in the absence of added erythropoietin was discovered (Johnson and Metcalf, 1977). In the following decades, numerous studies contributed to the identification and cloning of IL-3 (Ihle *et al.*, 1983; Prystowsky *et al.*, 1984). Today, it is widely

acknowledged that IL-3 plays an important role in regulating the proliferation, differentiation and survival of haematopoietic cells (Lee *et al.*, 1982).

The biological effects of BCR/ABL have been shown to overlap with IL-3, for example chronic administration of IL-3, or direct expression of IL-3 in mouse bone marrow by retroviral gene transfer leads to a hyperplasia similar to that observed in the chronic phase of CML. Moreover, it has also been shown that progenitor CD34+ cells from some CML patients can survive and proliferate *in vitro* in the absence of exogenous growth factors (Bedi *et al.*, 1994; Strife *et al.*, 1988). Similarly, the expression of p210BCR/ABL can abrogate IL-3 dependence in haematopoietic cell lines (Daley et al, 1988, Hariharan et al, 1988) and it is sufficient to induce G1-to-S phase transition, DNA synthesis and activation of cyclin-dependent kinases in cells that were arrested in G1 by growth factor deprivation (Cortez et al, 1997). Antisense transcripts of BCR/ABL abolished resistance to apoptosis after IL-3 withdrawal in BaF3 cells that p210 expression had rendered IL-3 independent.

Although BCR/ABL has been shown to activate many signal transduction pathways that overlap with those induced by cytokines, including the Ras/MAPK (Cortez et al, 1997) and JAK/STAT pathways (Ilaria et al, 1996), the exact mechanism by which BCR/ABL and interleukin IL-3 promote cell proliferation and survival has not been established.

This chapter details the work carried out to identify the regulation of the pRB pathway in mediating signals from IL-3 and BCR/ABL. Experiments described in the first parts of the chapter were designed to identify the potential targets of IL-3 and BCR/ABL in regulating the cell cycle progression, the mechanisms by which they are regulated and their roles. For this, the cell cycle profile of BCR/ABL expressing and non-expressing cells were analysed by FACS and the patterns

compared. Western and Northern blot experiments were used to study the expression and regulation of the control proteins upstream of pRB.

The final section looks into the possible upstream signalling pathways triggered by BCR/ABL and IL-3 to regulate cell proliferation and apoptosis.

3.2 The use of BaF3 cells as a model

The mouse pre-B cell line BaF3 requires IL-3 to proliferate as well as to overcome a default apoptotic program (Palacios and Steinmetz, 1985). BCR/ABL has been shown to be able to protect BaF3 cells from undergoing G1 cell arrest and subsequent apoptosis following IL-3 withdrawal (Daley and Baltimore, 1988). I investigated the proliferative signals derived from BCR/ABL and IL-3 in BaF3 cells and BaF3 cells stably expressing BCR/ABL (i.e. BaF3-p210) in the absence or presence of IL-3. The cells were split in fresh medium containing 10% (v/v) WEHI-3B conditioned medium as a source of IL-3 at a density of $1.5x10^6$ cells/ml. After 24 hours, half of each cell line was transferred to fresh media containing 10% (v/v) WEHI-3B conditioned medium while the other half was transferred to fresh media lacking the IL-3 supplement. After 24 hours the cells were harvested and stained for FACS analysis. Cell cycle analysis after propidium iodide staining (Figure 3.1) indicated that, in the presence of IL-3, both the parental and the BCR/ABL transformed BaF3 cells proliferated normally with no detectable sign of cell cycle arrest or apoptosis. In contrast, a significant difference was observed in the IL-3 deprived cells. The results revealed that following the withdrawal of IL-3, the parental BaF3 cells accumulated at the G1 phase of the cell cycle, before undergoing apoptosis. After 24 hours, over 80% of the cells were arrested in G1, and nearly all of the cells displayed detectable signs of apoptosis (<2N DNA content) after 72h of IL-3 depletion (Figure 3.1b). However, none of the BaF3-p210 cells underwent cell cycle arrest or apoptosis following the withdrawal of IL-3, confirming that expression of BCR/ABL could indeed protect BaF3 from the G1 cell cycle arrest and subsequent apoptosis induced by IL-3 depletion.

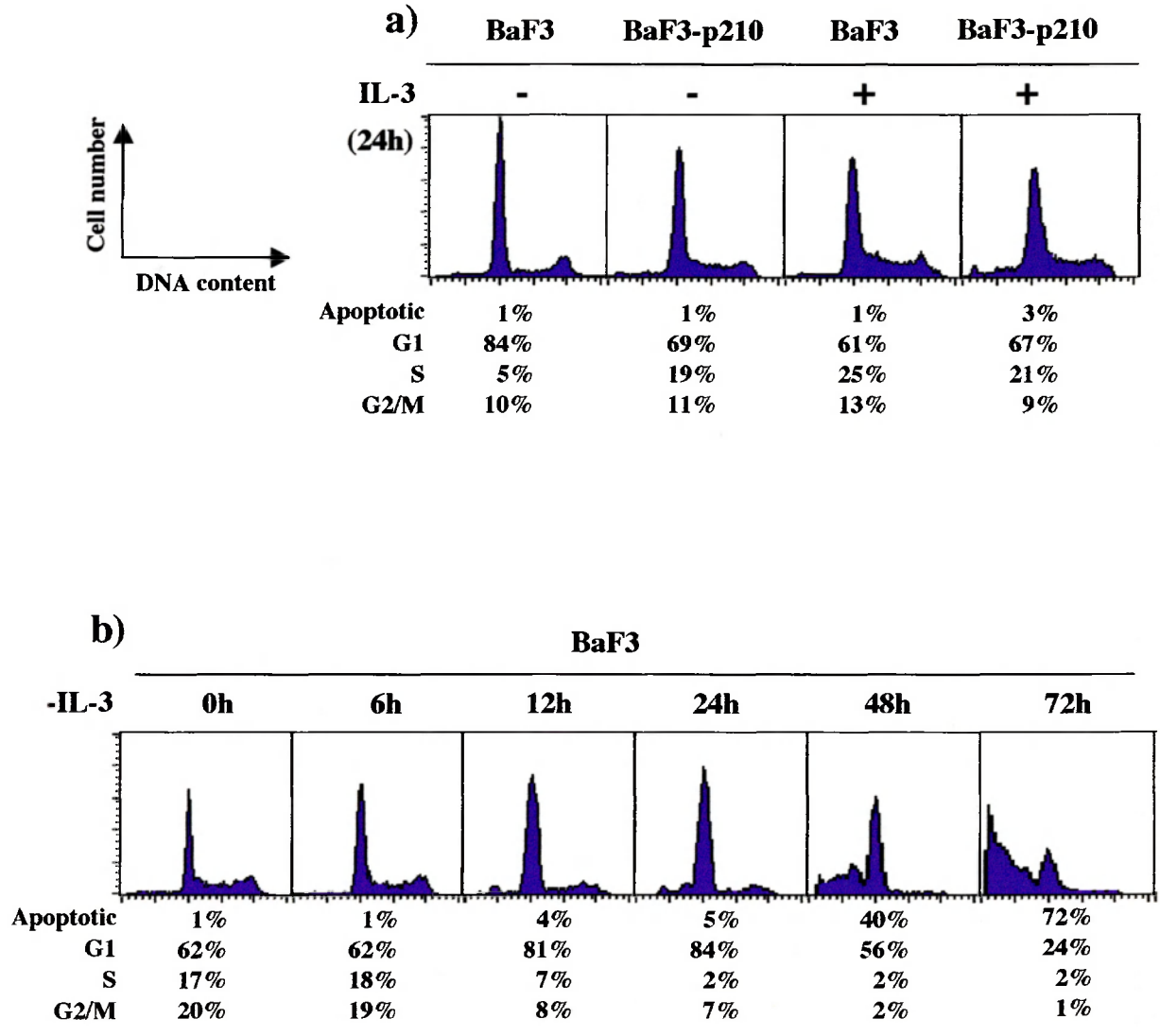

Figure 3.1. Effects of IL-3 and/or BCR/ABL on cell cycle progression and survival in BaF3 cells.

a) Cell cycle analysis of BaF3 and BaF3-p210 cells in the absence or presence of IL-3. Cells were cultured for 24h in the presence or absence of IL-3.
b) Cell cycle analysis of BaF3 cells after IL-3 withdrawal. Cells were harvested at 0h, 6h, 12h, 24h, 48h and 72h after IL-3 withdrawal.
Cell cycle progression was assessed by PI staining, as described in Chapter 2. The cell cycle profile is expressed as the number of cells against DNA content and the percentages of cells in each phase are indicated.

3.3 The expression and regulation of the cell cycle regulatory proteins.

To identify the targets of IL-3 and BCR/ABL in mediating cell survival and proliferation, the expression levels of molecules along the pRB pathway were examined in both BaF3 and BaF3-p210 cells cultured with and without IL-3 for 24 hours. BaF3 and BaF3-p210 cells were subjected to either 24 hours of IL-3 deprivation or left untreated. The cells were harvested and the lysates prepared with RIPA lysis buffer. The lysates (50μg) were fractionated on a 5% SDS-PAGE gel for the detection of high molecular weight proteins, such as pRB, p107, p130 and BCR/ABL, and on a 12% SDS-PAGE gel for the small proteins like cyclins and CDKs. Western blot analysis (Figure 3.2) showed that in IL-3 deprived BaF3 cells, the two pocket proteins, pRB and p107, were present in their respective faster migrating hypophosphorylated forms, while in the presence of IL-3 and/or BCR/ABL both were present at comparatively higher levels and in their hyperphosphorylated forms, which implies a derepression of E2F-dependent gene transcription and progression into S phase. In contrast, the expression of p130 was down-regulated in the presence of IL-3 or BCR/ABL Next, the expression patterns of cyclins, CDKs and CKIs, which are known to be responsible for controlling pocket protein phosphorylation, were studied. In IL-3-deprived BaF3 cells, the cyclins D2, D3, E and A were undetectable or present at very low levels, while their expression was detected at significant levels in the presence of IL-3 and/or BCR/ABL. In contrast to the cyclins, no dramatic difference was observed in the expression levels of their kinase partners, CDK2, CDK4 and CDK6 in both BaF3 and BaF3-p210 cells in the presence or absence of IL-3. It was concluded that IL-3 and BCR/ABL signals modulate the expression of the cyclins, but not their kinase subunits, CDKs.

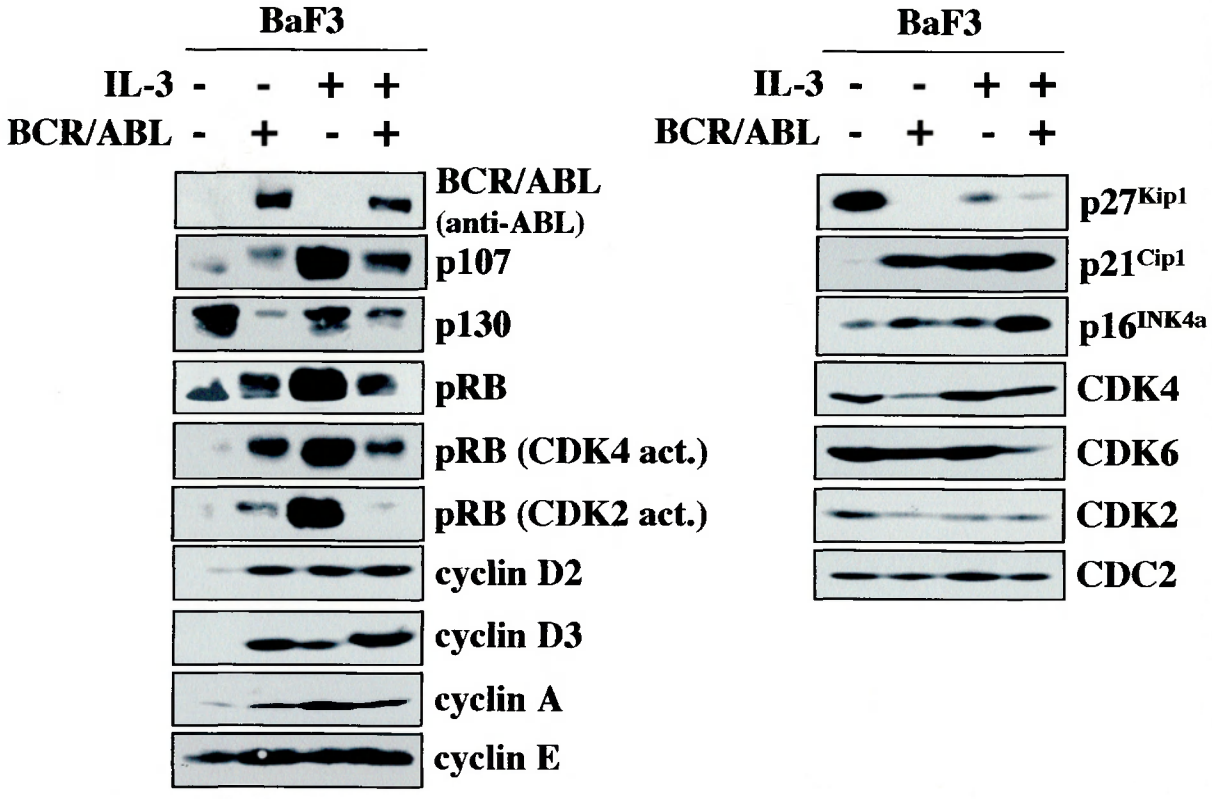

Figure 3.2. Western blot analysis of cell cycle related proteins in BaF3 cells in the absence or presence of IL-3 and/or BCR/ABL.

50μg of total cell extracts from BaF3 and BaF3-p210 cells cultured with and without IL-3 for 24h were separated by SDS-PAGE, blotted and probed with the antibodies indicated above. For the detection of BCR/ABL, p107, p130 and pRB, lysates were separated on a 5% SDS-PAGE. For all the other proteins a 12% SDS-PAGE gel was used.

I next investigated if the up-regulation of cyclin D and E expression was associated with the corresponding increases in their dependent kinase activity, which is responsible for pocket protein phosphorylation, using specific phospho pRB antibodies. pRB is the key physiological substrate for cyclin/CDKs, and specific sites are phosphorylated *in vivo* by distinct G1/S cyclin/CDKs. Therefore, antibodies that specifically recognise CDK4/6 or CDK2-phosphorylated residues on pRB were used to monitor the *in vivo* D- and E-type cyclin-dependent kinase activity in BaF3 cells in the presence or absence of IL-3 and BCR/ABL. This method has considerable advantages over *in vitro* kinase assays, particularly for studying CDK4/6 activity. The results obtained with *in vitro* kinase assays are frequently inconsistent, mainly due to phosphorylation by contaminating kinases and changes of CDK4/6 activities under the conditions used. Moreover, the immunoprecipitation procedures may also alter the kinase activities of the complexes. In contrast, using the CDK-specific anti-pRB antibodies permits the monitoring of the actual *in vivo* kinase activity of the cyclin and dependent kinase complexes in their physiological environment. Two pRB phospho-specific antibodies were used to recognise the phosphorylated forms of pRB: anti-pRB (Ser807/811), which recognises the CDK4/6 kinase-phosphorylated residues Ser807 and Ser811 and anti-pRB (Thr812), recognising pRB only when phosphorylated on Thr812 by CDK2. Figure 3.2 shows that pRB is phosphorylated at Ser807/811 at 24 h in the presence of IL-3 and/or BCR/ABL. In contrast, phosphorylated forms of pRB by CDK2 were hardly detectable in cells expressing BCR/ABL. This result might be explained by a later activation of cyclin E and A kinase activity in late G1 to S, presumably as a result of up-regulation of the expression of their respective cyclin and kinase subunits.

Since IL-3-deprivation induced a dramatic decrease in the enzymatic activity of the CDKs in BaF3 cells, despite the minimal effect in their protein expression, it

was of interest to study the expression of molecules involved in the control of the CDK activity. An important mechanism of inhibition of CDK activity is represented by the CKI superfamily, therefore their expression was examined. Interestingly, it was observed that only $p27^{Kip1}$ was up-regulated in IL-3 starved BaF3 cells and substantially down-regulated in the presence of IL-3 and/or BCR/ABL expression. $p21^{Cip1}$, another member of the Cip/Waf family of CKIs, and $p16^{INK\$a}$, a member of the INK family, were undetectable in IL-3 starved BaF3 cells, but abundantly expressed in response to IL-3 and/or BCR/ABL. These results suggested that $p27^{Kip1}$, but neither $p21^{Cip1}$ nor $p16^{INK4a}$, plays a role in repressing CDK activity in the BaF3 cells when deprived of growth and survival signals. Since the expression of cyclin E and CDK2 were not significantly modulated by BCR/ABL, this finding indicated that BCR/ABL could be targeting $p27^{Kip1}$ to modulate the activity of cyclin E/CDK2.

The results described above indicated that $p27^{Kip1}$, cyclin D2 and cyclin D3 might be important targets for IL-3 and BCR/ABL in regulating cell proliferation and/or survival. Thus, the IL-3 or BCR/ABL derived signals up-regulate cyclin D2 and D3 expression and down-regulate the expression level of the cyclin-dependent kinase inhibitor $p27^{Kip1}$, culminating in the induction of cyclin-dependent kinase activity, pocket protein hyperphosphorylation, activation of E2F activity and continued cell proliferation.

3.4 Modulation of cyclin D2, cyclin D3 and $p27^{Kip1}$ mRNA levels.

To explore the mechanisms by which IL-3 and BCR/ABL regulate cyclin D2 and D3 and $p27^{Kip1}$ expression, Northern blot analyses were performed on BaF3 and BaF3-p210 cells in the presence or absence of IL-3 (Figure 3.3). I observed an up-regulation of cyclin D2 mRNA levels and a down-regulation of $p27^{Kip1}$ mRNA in the presence of IL-3 and/or BCR/ABL expression. In a paradox with the results obtained at the protein level, the level of cyclin D3 mRNA was largely unchanged with or without IL-3, but was down-regulated in the BaF3 cells expressing BCR/ABL.

Collectively, these observations suggested that cyclin D2 and $p27^{Kip1}$ expression in response to IL-3 and/or BCR/ABL are regulated, at least partially, at the transcriptional level. In contrast, the expression of cyclin D3 appears to be regulated by a different mechanism.

To discriminate possible artefacts associated with the selection of BaF3 cells stably expressing BCR/ABL, two different approaches were followed: firstly, a BaF3 cell line with tetracycline-dependent BCR/ABL expression (TonB210.1) was used, and secondly, BCR/ABL activity in BaF3-p210 cells was inhibited using the drug STI571.

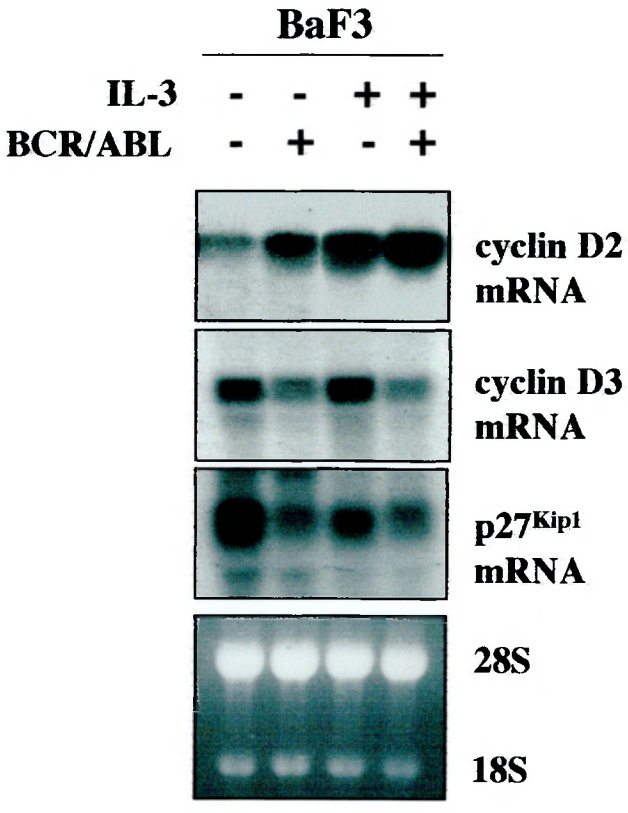

Figure 3.3. Northen blot analysis of cyclin D2, cyclin D3 and $p27^{Kip1}$ in BaF3 cells in the absence or presence of IL-3 and/or BCR/ABL.

BaF3 and BaF3-p210 cells were cultured with and without IL-3 for 24h. Equal loading of total RNAs was confirmed by staining with ethidium bromide.

3.5 Expression of Cyclin Ds and $p27^{Kip1}$ is associated with BCR/ABL expression.

TonB210.1 cells are IL-3 dependent, but demonstrate IL-3 independence in the presence of the tetracycline analogue doxycycline (Klucher *et al.*, 1998). This cell line, derived from BaF3 cells, was generated using the control elements of the tetracycline resistance operon encoded in Tn10 of *Escherichia coli* to regulate gene expression. As a result, TonB210.1 cells express BCR/ABLp210 in response to the addition of doxycycline.

Initially, the TonB210.1 cells were starved of IL-3 to induce cell arrest in the G1 phase, and 48 hours later doxycycline was added to stimulate BCR/ABL expression and entry in the cell cycle. Contrary to expectations, the cells did not progress though the cell cycle after doxycyclin stimulation. Trypan blue staining indicated that after 48 hours of IL-3 depletion, a large proportion of the cells was already dead. It is likely that the remaining population had already activated irreversible apoptotic pathways.

A new strategy was then designed: the cells were cultured in the absence of IL-3 for 48 hours and treated with doxycycline to induce BCR/ABL expression at 3h, 12h, 24h and 48 hours prior to harvesting. All the cells were collected at 48 hours and stained for FACS analysis. Cell cycle analysis after propidium iodide staining (Figure 3.4) showed that after 48 hours of IL-3 starvation, the majority of the non-induced TonB210.1 cells accumulated at G1 phase of the cell cycle (>70%). The same profile was observed for those cells treated with doxycycline for both 3 and 12 hours. However, this arrest could be partially abrogated in those cells treated with doxycycline for 24 hours: there was a significant reduction of cells in G1 and a concomitant increase of cells in S and G2/M phase. Cells stimulated for 48 hours presented the same profile as those cultured in the presence of IL-3.

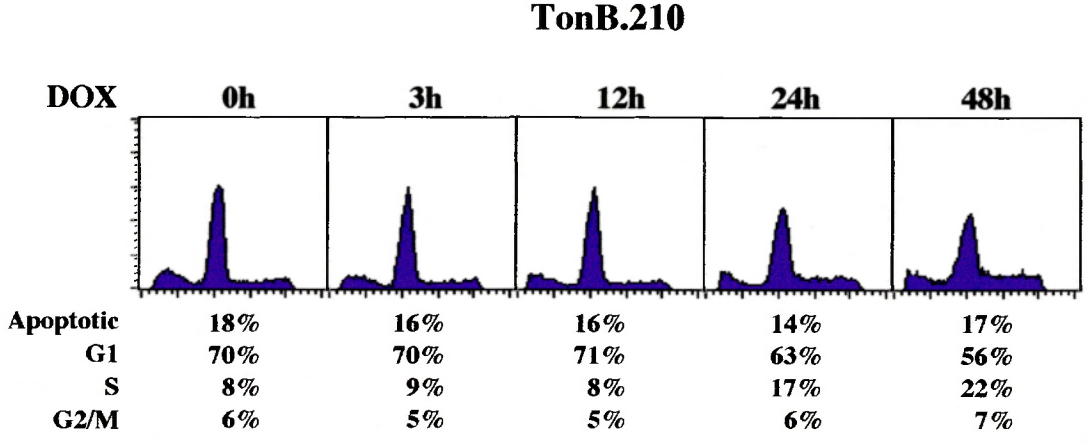

Figure 3.4. BCR/ABL expression promotes progression into cell cycle.

TonB210.1 cells were cultured in absence of IL-3 and treated with doxycycline (1μg/ml) to induce BCR/ABL expression at 0h, 3h, 12h, 24h and 48h prior to harvesting.
Cell cycle progression was assessed by PI staining, as described in Chapter 2.

The expression of BCR/ABL was analysed by Western blotting (Figure 3.5). The induction of BCR/ABL expression upon addition of doxycycline was first detectable by 24 hours, concurrent with the cells re-entering S and G2/M phases from G1. By the same time, the expression of cyclin D2, D3 and E was induced, while the expression levels of their dependent-kinases, CDK4, 6 and 2, remained largely unchanged after the induction of BCR/ABL expression. Significantly, the up-regulation of the cyclin D2, D3 and E was associated with an increase in cyclin D – and E-dependent kinase activity. The three pocket proteins became hyperphosphorylated at 24 hours, and thus decreased their mobility in SDS-PAGE.. Again, the expression of the CKI $p27^{Kip1}$ was inversely correlated to that of BCR/ABL. $p27^{Kip1}$ accumulated in the absence of BCR/ABL expression, concomitant with cell cycle arrest and pocket protein hypophosphorylation. However, after 24 hours of doxycycline treatment, a dramatic down-regulation in $p27^{Kip1}$ levels was observed.

After 48 hours, the expression of BCR/ABL reached its maximum, and was accompanied by an intensified up-regulation of cyclin D2 and down-regulation of $p27^{Kip1}$, p107 and p130 expression and their hyperphosphorylated forms continued to be up-regulated, whereas pRB was present at lower levels.

These results confirm that BCR/ABL can overcome the cell cycle arrest induced by IL-3 withdrawal by modulating the expression of cyclin D2, D3 and $p27^{Kip1}$.

The Northern blot analysis revealed that the cyclin D2 mRNA level was up-regulated, while the $p27^{Kip1}$ mRNA level was down-regulated, in response to BCR/ABL induction (Figure 3.6). Moreover, they showed that the kinetics of cyclin D2 and $p27^{Kip1}$ mRNA expression correlated with those of their respective proteins. Consistent with the previous results, the level of cyclin D3 mRNA was decreased upon BCR/ABL induction.

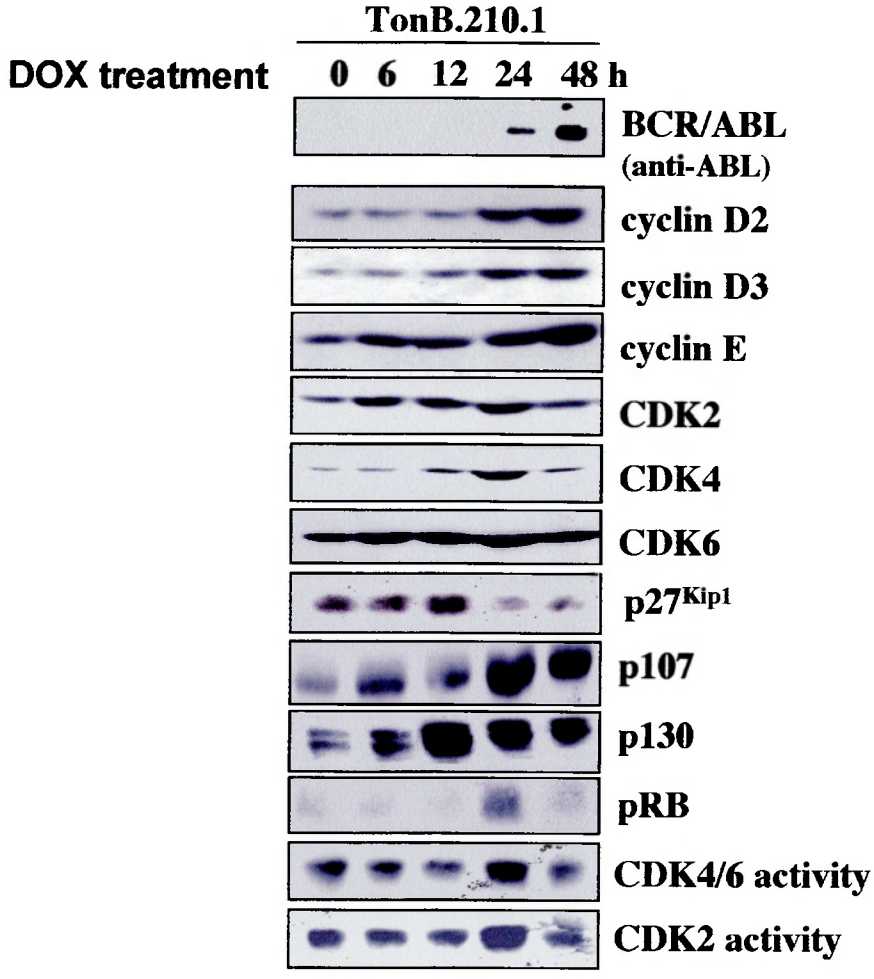

Figure 3.5. Effects of the induction of BCR/ABL expression cells in the expression of cell cycle related proteins in TonB210.1.

Cells were cultured in the absence of IL-3 for 48h and treated with doxycycline (1μg/ml) to induce BCR/ABL expression at 0h, 3h, 10h, 24h and 48h prior harvesting. 50μg of total cell extracts were separated by SDS-PAGE, blotted and probed with the antibodies indicated above.

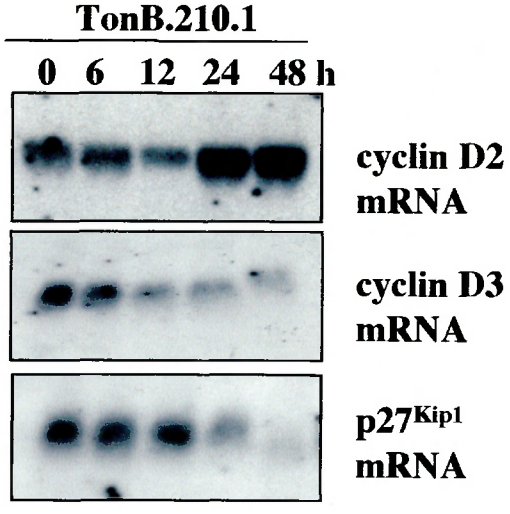

Figure 3.6. Northen blot analysis of cyclin D2, cyclin D3 and $p27^{Kip1}$ after induction of BCR/ABL expression in TonB210.1 cells.

Cells were cultured in the absence of IL-3 for 48h and treated with doxycycline (1μg/ml) to induce BCR/ABL expression at 0h, 3h, 10h, 24h and 48h prior to harvesting.

3.6 Effects of the inhibition of BCR/ABL activity in BaF3-p210 cells using the specific inhibitor STI571

STI571, a 2-phenylaminopyrimidine derivative, has been shown to selectively inhibit the tyrosine kinase activity of c-ABL and BCR/ABL(Druker *et al.*, 1996). It has been demonstrated that the drug STI571 can specifically block the proliferation of BCR/ABL-positive cell lines and tumors and induce these cells to undergo apoptosis (Beran *et al.*, 1998; Deininger *et al.*, 1997). BaF3-p210 cells were deprived of IL-3 and treated with 25μM STI571 for 24 hours. The cells were collected and both the cell cycle profiles and the expression of cell cycle regulatory molecules were analysed.

Inhibition of BCR/ABL with STI571 resulted in an arrest of the cells at G1 phase. After 24 hours of treatment with STI571, 85% of the BaF3-p210 cells were arrested in G1 compared to 53% for the untreated cells. Interestingly, this arrest was overcome by IL-3, suggesting that both molecules affect the same signalling pathways (Figure 3.7). In effect, cells cultured in the presence of IL-3 proliferate normally with no detectable sign of cell cycle arrest or apoptosis.

It is interesting to notice that, although both molecules induce cell cycle progression, cell proliferation was not enhanced by the combination of IL-3 and BCR/ABL. This lack of synergy between the molecules supports the hypothesis that both BCR/ABL and IL-3 activate similar signalling pathways and hence a threshold is imposed in their biological effects by the maximum activation level of their substrates.

Western blot results showed that the inhibition of BCR/ABL by STI571 resulted in a down-regulation of the expression of Cyclin D2 and D3, but induced the expression of $p27^{Kip1}$ (Figure 3.8). This was accompanied by the down-regulation of both cyclin D-CDK4/6 and cyclin E-CDK2 activity, as revealed by the specific

anti-phospho-pRB antibodies. Consistent with previous results, the pocket proteins pRB and p107 were down-regulated and hypophosphorylated following STI571 treatment, but the p130 expression was induced in response to STI571.

The Northern blot results confirmed the regulation at the transcriptional level of both cyclin D2 and $p27^{Kip1}$ (Figure 3.9). However, despite the cyclin D3 protein level being significantly down-regulated by STI571, the cyclin D3 mRNA level was not dramatically affected. This suggests that the expression of cyclin D3 is regulated by BCR/ABL at the post-transcriptional level.

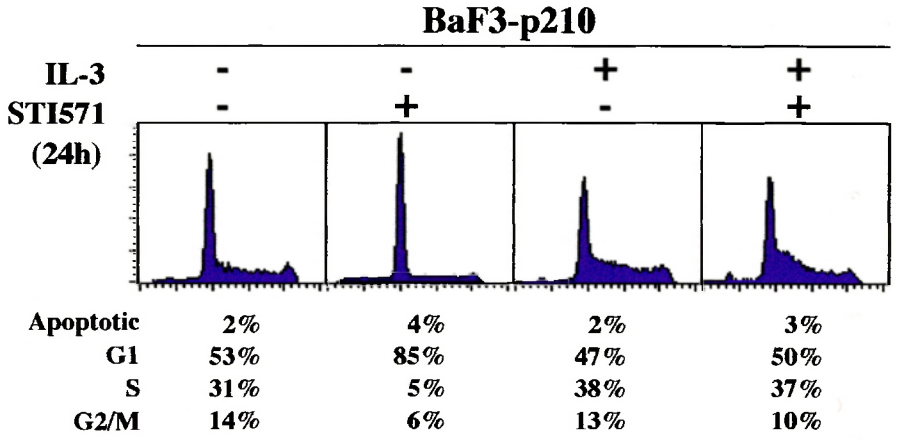

Figure 3.7. STI571 abrogates the BCR/ABL-mediated rescue from G1 arrest in BaF3-p210 cells.

Cells were incubated with STI571 (25μM) for 24h in the presence or absence of IL-3. Cell cycle progression was assessed by PI staining as described in Chapter 2.

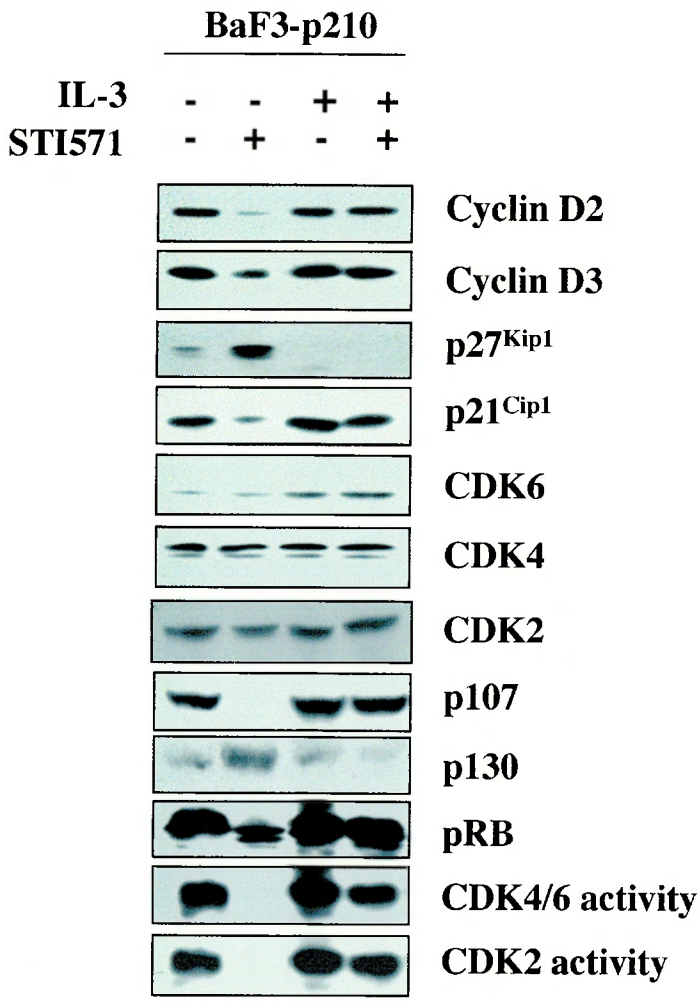

Figure 3.8. Effects of STI571 on the expression of cell cycle related proteins in BaF3-p210 cells.

Cells were incubated for 24h with STI571 (25μM) in the absence or presence of IL-3. 50μg of total cell extracts were separated by SDS-PAGE, blotted and probed with the antibodies indicated above.

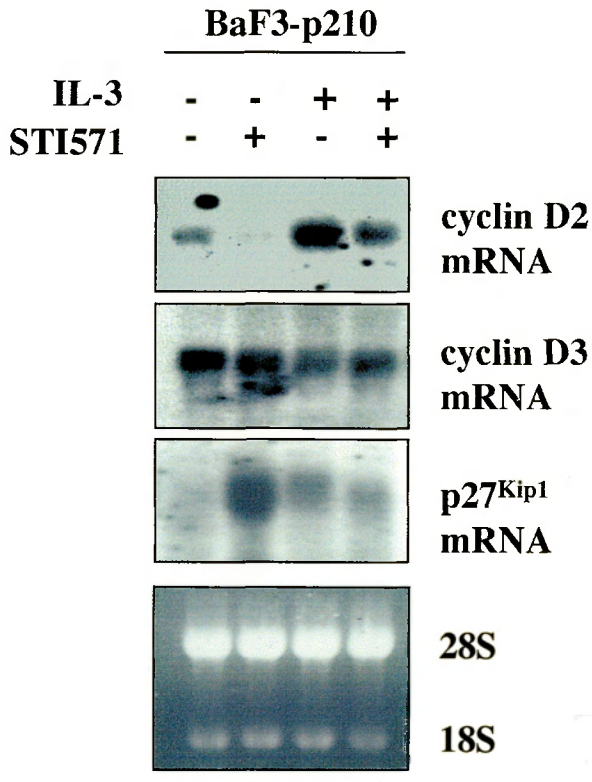

Figure 3.9. Northen blot analysis of cyclin D2, cyclin D3 and $p27^{Kip1}$ in BaF3-p210 cells after treatment with STI571.

Cells were incubated for 24h with STI571 (25μM) in the absence or presence of IL-3. Equal loading of total RNAs was confirmed by staining with ethidium bromide.

3.7 Cell cycle analysis of BaF3 and BaF3-p210 cells after overexpression of cyclin D2.

Having observed that the expression of cyclin D2 in BaF3 and BCR/ABL transformed BaF3 cells was correlated with a proliferating status, it was of interest to investigate whether cyclin D2 could induce cell growth and/or provide protection from cell death. To determine if cyclin D2 plays an important role in mediating proliferation and survival of these cells, I analysed whether exogenous expression of cyclin D2 could abolish the G1 cell cycle arrest and apoptosis induced by IL-3 withdrawal and STI571 treatment in BaF3 and BaF3-p210 cells, respectively. Stable transfectants of BaF3 and BaF3-p210 cells, constitutively over-expressing cyclin D2, were generated. The full-length mouse cyclin D2 cDNA was cloned into the BamH1 cloning site of the pBABE-puro expression vector, a plasmid containing a puromycin resistance gene. The plasmid was transfected into BaF3 and BaF3-p210 cells using electroporation. Cells transfected with the empty expression vector were used as a control.

To test the ability of cyclin D2 to induce proliferation and/or protect cells from apoptosis, IL-3 deprivation was used to induce apoptosis. BaF3, BaF3-p210 cells and their cyclin D2-transfected clones were cultured in the absence of IL-3 for 24 hours. BaF3-p210 and BaF3-p210-cyclin D2 cells were additionally treated with 25μg/ml of STI571 for the same period of time. Cell cycle analysis by PI staining revealed that after 24 hours of IL-3 deprivation, 82% of the BaF3 cells were arrested at the G1 phase of the cell cycle compared to 72% for BaF3-cyclin D2 cells (Figure 3.10). Similarly, 72% of BaF3-p210 cells were arrested in G1 after 24 hours of STI571 treatment, while the BaF3-p210 cells over-expressing cyclin D2 proliferated normally (Figure 3.11).

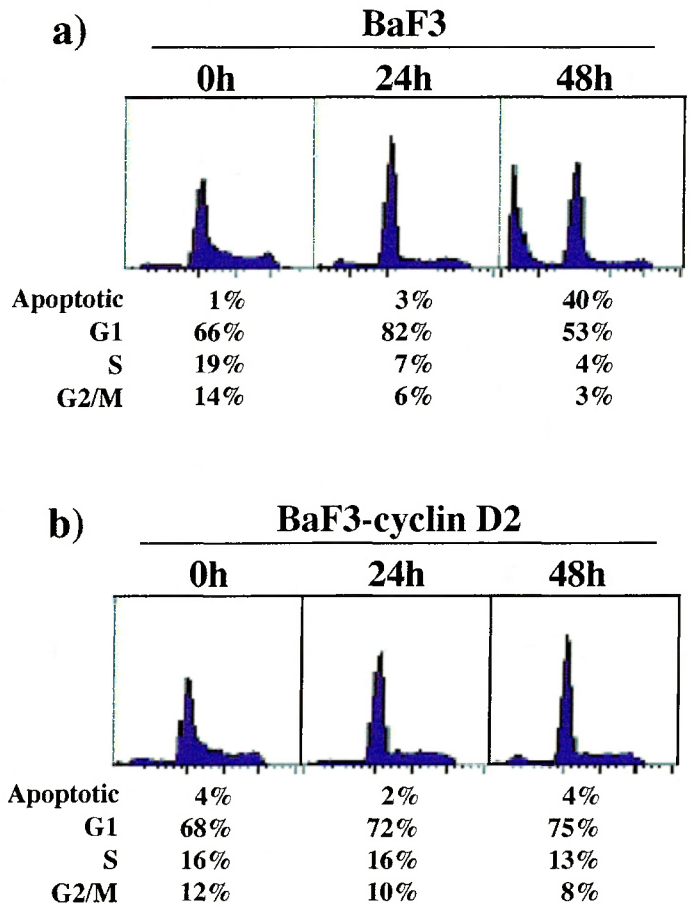

Figure 3.10. Ectopic expression of cyclin D2 overcomes the G1 arrest and apoptosis induced by IL-3 withdrawal in BaF3 cells.

a) Cell cycle analysis of BaF3 cells at 0h, 24h and 48h after IL-3 withdrawal.
b) Cell cycle analysis of BaF3-cyclin D2 at 0h, 24h and 48h after IL-3 withdrawal.
Cells cultured in the absence of IL-3 were harvested at 0h, 24h and 48h and analysed as described in chapter 2.

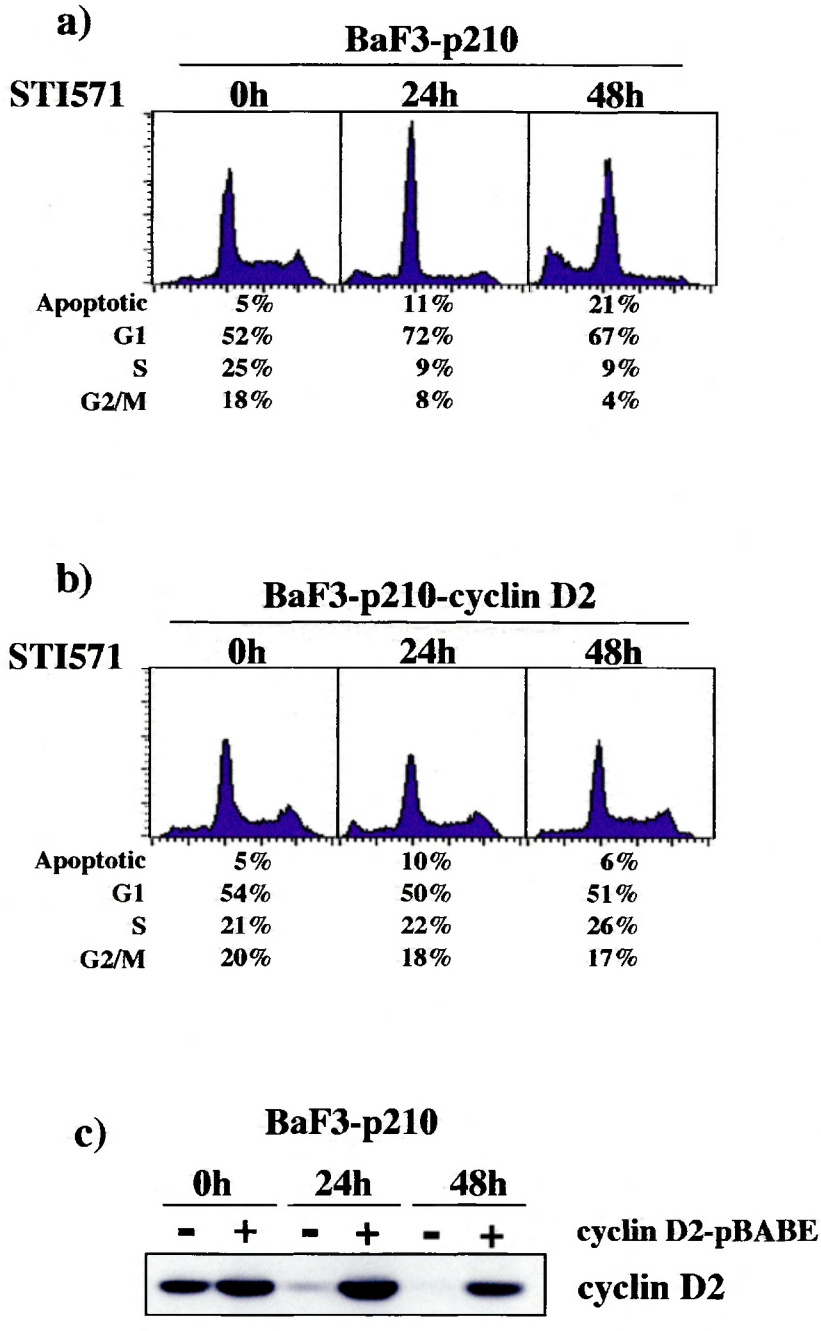

Figure 3.11. Ectopic expression of cyclin D2 overcomes the G1 arrest and apoptosis induced by STI571 treatment in BaF3-p210 cells.

a) Cell cycle analysis of BaF3-p210 cells at 0h, 24h and 48h after STI571 treatment.
b) Cell cycle analysis of BaF3-p210-cyclin D2 at 0h, 24h and 48h after STI571 treatment.
Cells were cultured in the absence of IL-3 and treated with 25μM of STI571. Cells were harvested at 0h, 24h and 48h and analysed as described in chapter 2.
c) The expression of cyclin D2 in BaF3-p210 cells after STI571 treatment was analysed by Western blotting.

The difference between the parental cells and the cyclin D2-expressing cells continued and increased over the following 24 hours. After 48 hours only 7% of the BaF3 cells remained cycling against over 20% of the cyclin D2 transfected cells. Moreover, 40% of the BaF3 cells underwent apoptosis (sub-G1 phase), whereas only 4% of BaF3-cyclin D2 were dead. BaF3-p210 cells showed a drastic reduction in their proliferating population, while BaF3-p210-cyclin D2 cells continued to grow normally.

To verify this phenomenon further, Annexin V staining was performed in BaF3-p210 and BaF3-p210-cyclin D2 cells (Figure 3.12). Annexin V is an anticoagulant protein that preferentially binds negatively charged phospholipids. Early in the apoptotic process, the phospholipid asymmetry between the inner and outer leaflets of the plasma membrane is disrupted leading to the exposure of phosphatidylserine (PS) on the outer leaflet. Annexin V binding to PS is calcium dependent and of very high affinity. The binding of Annexin V-FITC to cells permits a differentiation of apoptotic cells (Annexin V positive) from non-apoptotic cells (Annexin V negative). The data obtained confirms that ectopic expression of cyclin D2 can prevent the cell arrest and apoptosis induced by STI571 in BaF3-p210 cells.

It is interesting to notice that although both techniques revealed the ability of cyclin D2 to induce cell proliferation and to protect cells from apoptosis, they show significantly differing percentages of viable and apoptotic cells. This could be explained by the higher sensitivity of Annexin V staining compared to PI staining for the detection of apoptotic cells. Whereas Annexin V fully differentiates apoptotic from viable cells, analysis with propidium iodide staining might fail to distinguish these populations, for example early apoptotic cells whose DNA content has not yet decreased from 2N or cell clumps.

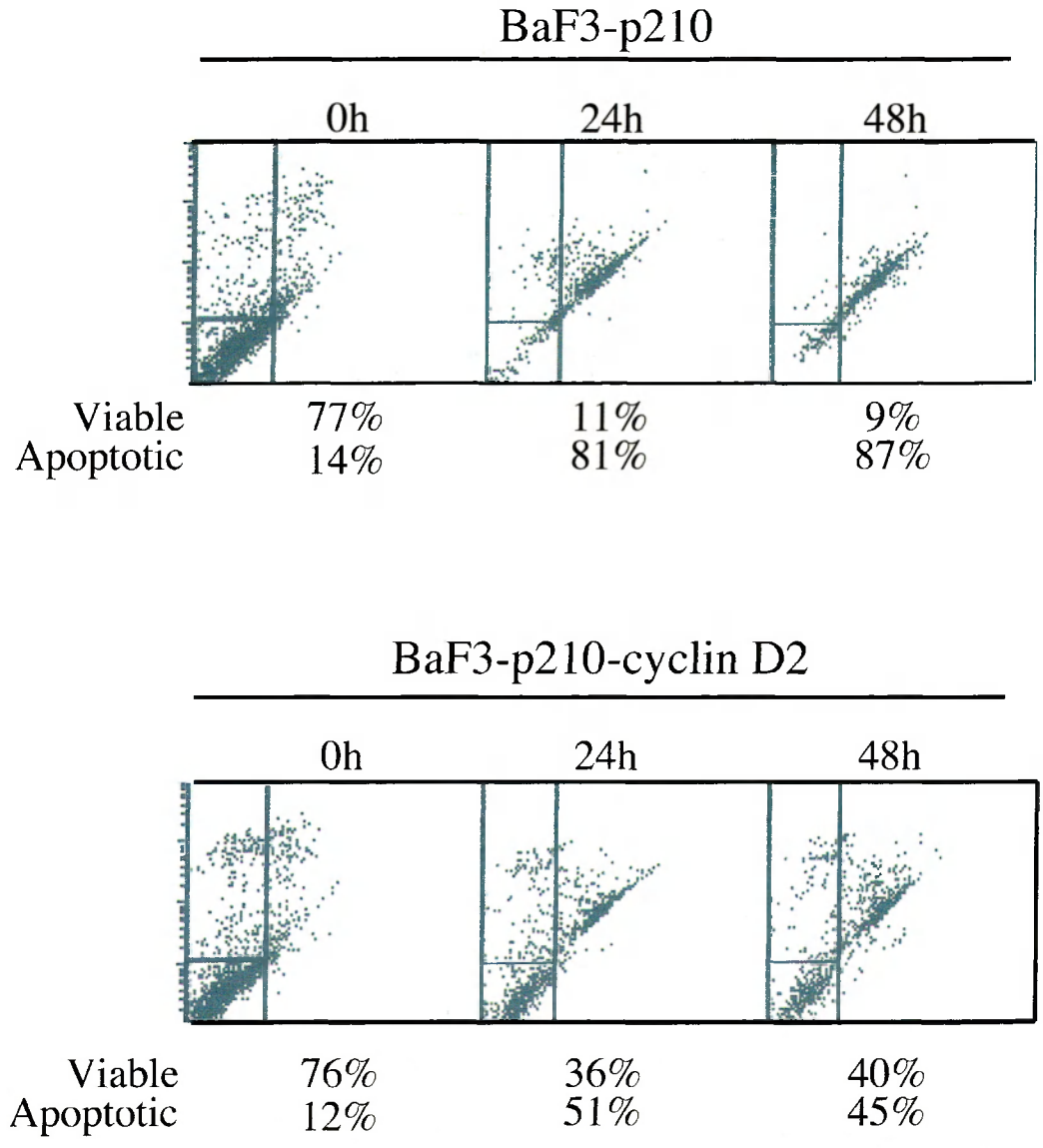

Figure 3.12. Effects of overexpression of cyclin D2 on cell cycle progression and survival of BaF3-p210 cells.

BaF3-p210 and BaF3-p210-cyclin D2 cells were IL-3 deprived for 48h. Cells were harvested at 0, 24 and 48h and stained with Annexin V and propidium iodide as described in chapter 2 for cell viability. The x-axis reflects the log Annexin V-FITC fluorescence and the y-axis reflects the PI fluorescence. Viable cells are negative for both Annexin V and PI (lower left quadrant). Early apoptotic cells bind Annexin V (lower right quadrant) and late apoptotic cells also bind PI as a consequence of membrane permeabilisation (upper right quadrant). The percentages of viable and apoptotic cells revealed by FACS analysis are shown.

To confirm that the relative resistance of cyclin-D2 transfected BaF3 and BaF3-p210 cells to cell death was related to the presence of cyclin D2, the expression of the latter was analysed by Western blotting (Figure 3.11c). The levels of cyclin D2 in the transfected BaF3-p210 cells remained constant during IL-3 deprivation and STI571 treatment. In contrast, the expression of cyclin D2 in the parental cells drastically decreased after 24 hours of treatment.

Under normal tissue culture conditions, BaF3 and BaF3-p210 cells stably transfected with pBABE-puro-cyclin D2 showed a growth advantage over the parental cells, suggesting that the procedure of creating stable transfectants could involve the selection of the best growing cells. However, this enhanced growth phenotype was shown to be specific for the cyclin D2 transfected cells, as the pBABE-puro transfected cells presented the same growing profile as the parental cells. To assure that the changes observed were specifically due to Cyclin D2 and not due to unrelated mutations in the cultured cell lines, the same experiments were planned in transient transfectants. Unfortunately, the transient transfection system was unsuitable for quantitative analysis since the BaF3 cells are very sensitive to electroporation and require a long period of recovery before IL-3 removal is indicated. In addition, the low transfection efficiency will tend to mask the resistance to apoptosis.

The results obtained indicate that cyclin D2 is required and sufficient to induce cell cycle progression in BaF3 and BaF3-p210 cells.

3.8 Expression of $p27^{Kip1}$ induces cell cycle arrest and apoptosis in BaF3 cells.

It was postulated that the induction of $p27^{Kip1}$ was sufficient to cause cell cycle arrest of BaF3 cells and to induce apoptosis. The initial evaluation of this hypothesis was undertaken by transfecting $p27^{Kip1}$ in BaF3 cells using electroporation. However, this method proved to be unsuitable for the analysis of $p27^{Kip1}$ apoptotic effect because it was not possible to generate stable clones and the cell mortality in transient transfections due to either the electroporation process or the introduction of $p27^{Kip1}$ could not be distinguished. A different protocol was then adopted for introducing $p27^{Kip1}$ protein in BaF3 cells. In 1988, the human immunodeficiency virus (HIV) TAT protein was discovered to be able to cross cell membranes (Frankel and Pabo, 1988; Green and Loewenstein, 1988). This property has been exploited to transduce heterologous proteins into cells by chemically cross-linking a 36-amino acid domain of TAT to them (Fawell *et al.*, 1994). Cells have been shown to take up proteins fused to the 11-amino acid transduction domain of the (HIV) TAT protein (Nagahara *et al.*, 1998). To investigate the functional significance of $p27^{Kip1}$, BaF3 and BaF3-p210 cell lines were transduced with biologically active TAT-$p27^{Kip1}$ or with TAT-mutant $p27^{Kip1}$ (TAT-$p27^{Kip1}$ KK) and TAT-eGFP as controls (Nagahara *et al.*, 1998) (Figure 3.10). Cells incubated with TAT-eGFP and TAT-mutant $p27^{Kip1}$ proliferated normally, but the majority of the $p27^{Kip1}$-transduced BaF3 displayed sub-G1 (<2 N) DNA content, indicative of apoptosis (Figure 3.13). The ability of wild-type $p27^{Kip1}$, but not mutant $p27^{Kip1}$, to promote apoptosis in BaF3 cells, even in the presence of IL-3, was confirmed by annexin V staining (Figure 3.14). These observations indicated that similar to IL-3 depletion, expression of $p27^{Kip1}$ alone is sufficient to induce apoptosis in BaF3 cells. The propidium iodide staining also showed that the TAT $p27^{Kip1}$-transduced BaF3-p210 cells ceased growth and

accumulated in G1, whereas control cells multiplied normally. Interestingly, only low levels of apoptosis were detected in these cells expressing BCR-ABL (Figure 3.13). The presence of TAT-$p27^{Kip1}$ fusion proteins in the BaF3 and BaF3-p210 cells was confirmed by Western blotting for $p27^{Kip1}$ protein (Figure 3.13b).

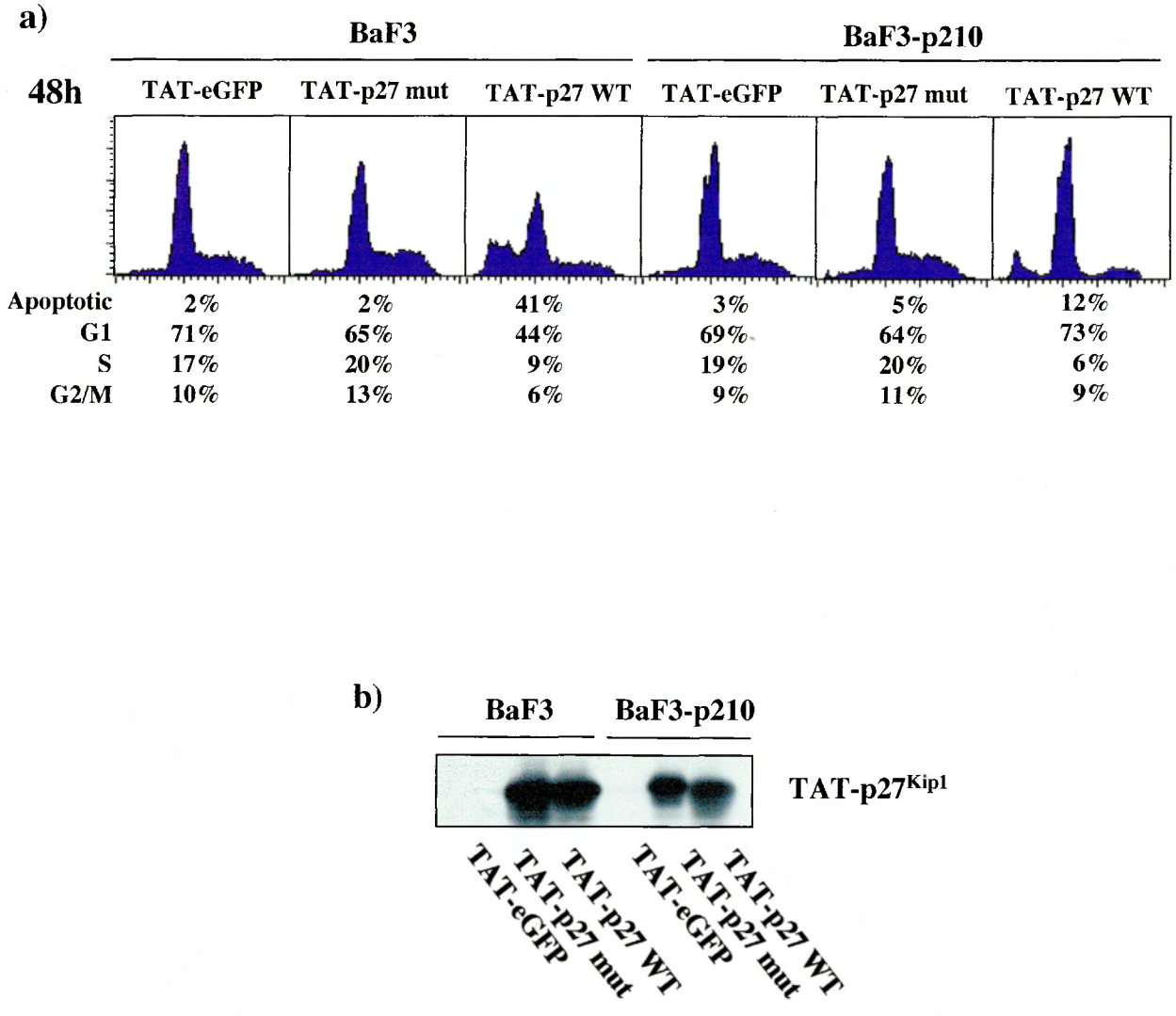

Figure 3.13. Effects of overexpression of $p27^{Kip1}$ on cell cycle progression and survival on BaF3 and BaF3-p210 cells.

Cells were transduced with TAT-eGFP, TAT-p27 (KK mutant) or TAT-p27 WT for 48h.

a) Cell cycle progression was assessed by propridium iodide staining as described in Chapter 2.

b) The expression of TAT-p27 proteins was monitored by immunoblotting.

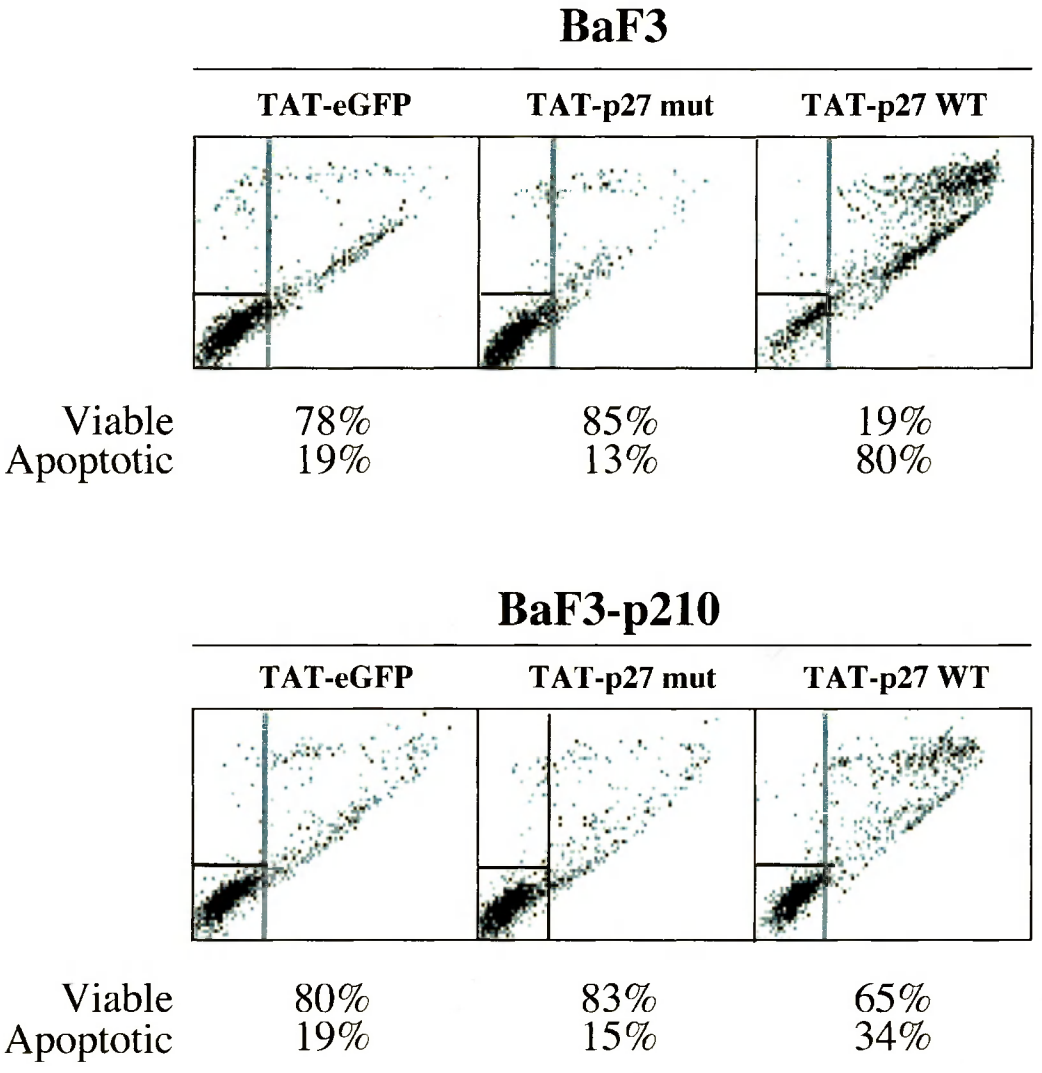

Figure 3.14. Effects of overexpression of $p27^{Kip1}$ on cell cycle progression and survival on BaF3 and BaF3-p210 cells.

Cells were transduced with TAT-eGFP, TAT-$p27^{Kip1}$ (KK mutant) or TAT-$p27^{Kip1}$ WT for 48h. Cells were harvested at 0h, 24h and 48h and stained with Annexin V and propidium iodide as described in chapter 2 for cell viability. The percentages of viable and apoptotic cells revealed by FACS analysis are shown.

3.9 Regulation by phophatidyl-inositol-3 Kinase (PI 3-K)

Since IL-3 stimulates a cascade of membrane and cytoplasmic protein phosphorylation, it was hypothesised that abrogation of IL-3-dependence in BaF3 cells by BCR/ABL might result from phosphorylation and activation of the same proteins involved in IL-3 signal transduction. Having established cyclin D2, D3 and $p27^{Kip1}$ as important targets of BCR/ABL and IL-3, I tried to identify the pathway involved in the transduction of the signal between BCR/ABL and its downstream effectors.

Both MAPKinase and PI 3-K pathways are known to be active in BCR/ABL transformed cells and can be interrupted by specific inhibitors. PI 3-K is involved in a variety of cellular responses, including cell growth, survival, metabolism, differentiation and membrane trafficking (Leevers *et al.*, 1999). Various downstream mediators of PI 3-Kinase activity have been identified (Cross *et al.*, 1995; Franke *et al.*, 1995). Among them, the serine/threonine kinase AKT (also referred as PKB) is thought to have a role in proliferative and anti-apoptotic cell responses (Dudek *et al.*, 1997). AKT is encoded by the *Akt* proto-oncogene and is defined by a NH_2 terminal regulatory domain of protein-protein interaction that contains a PH domain (Marte and Downward, 1997). Previous studies have shown that the activity of AKT is regulated directly by products of PI 3-K that bind to the AKT PH domain (Franke *et al.*, 1997; Klippel *et al.*, 1997). AKT has been shown to activate the p70 ribosomal protein S6 ($p70^{S6K}$) and suppress the pro-apoptotic function of the Bcl2 family member BAD (Datta *et al.*, 1997; del Peso *et al.*, 1997), the protease caspase 9 (Cardone et *al.*, 1998) and the FH family member FKHRL1 (Brunet et *al.*, 1999: del Peso et *al.*, 1999). Upon activation by extracellular signals, primarily in a PI 3K-dependent manner, AKT translocates to the plasma membrane and binds through its PH domain to the lipid products of PI

3-K. Once in the plasma, AKT is fully activated by phosphorylation at specific residues (Marte and Downward, 1997). Two major phosphorylation sites have been identified: the threonine 308 and serine 473 (Meier *et al.*, 1997).

Western blots performed in BaF3 and BaF3-p210 cells in presence or absence of IL-3 showed a correlation between the phosphorylation of AKT and the presence of IL-3 and/or the expression of BCR/ABL (Figure 3.15). Using an activation specific-antibody which recognises the Thr308 phosphorylated kinase-active form of AKT, it was observed that AKT is constitutively activated under condition of IL-3 deprivation in BaF3-p210 cells. Also, activation of AKT was induced by BCR/ABL expression in Tonb210 cells. These results suggest that modulation of D type cyclins and $p27^{Kip1}$ protein levels may occur through constitutive activation of the PI 3-Kinase pathway. To investigate the potential role of PI 3-Kinase in mediating BCR/ABL signals, the effects of a specific inhibitor of the PI 3-Kinase activity, LY294002, on BaF3 cells and BaF3-P cells were studied. FACS analysis after 24 hours of treatment showed a clear arrest in G1 for both BaF3 and BaF3-p210 cells, independent of the presence of IL-3 (Figure 3.16). The same data were obtained using another specific inhibitor of PI 3-K, wortmannin, which is structurally unrelated to LY294002 (data not shown). Western blot results confirm the down-regulation of cyclin D2 and the up-regulation of $p27^{Kip1}$ in BaF3-p210 cells after 24 hours of treatment with LY294002 (Figure 3.17). Interestingly, no major difference was observed in the expression of cyclin D3, suggesting that cyclin D3 levels may be regulated, at least partially, through another pathway or further downstream on the same signalling pathway. The expression of CDK4 and CDK6 was not affected by LY294002 treatment. Consistent with previous results, p107 was down-regulated while p130 expression was induced in response to LY294002. LY294002 treatment resulted in a dephosphorylation of AKT whilst total levels remained constant.

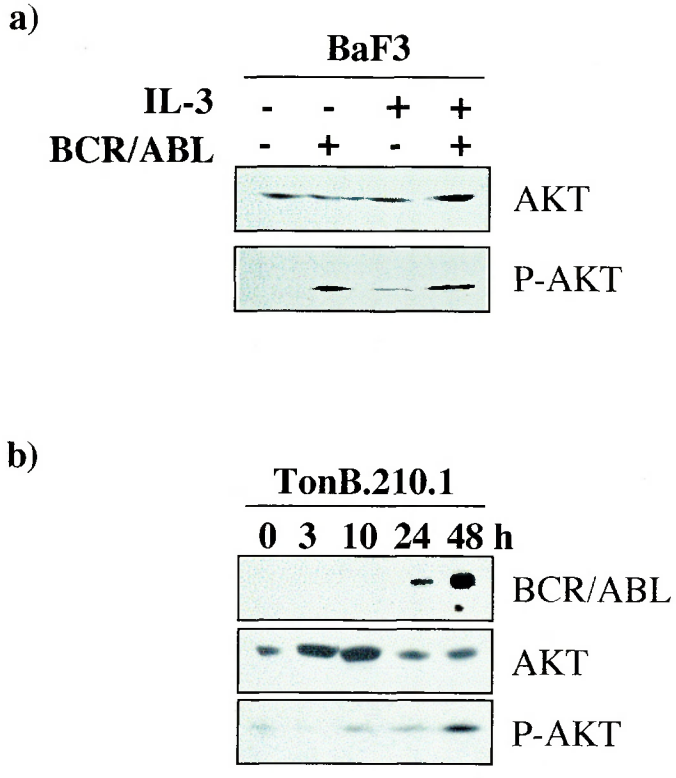

Figure 3.15. AKT is activated in response to BCR/ABL.

The expression of total AKT and its phosphorylated form was analysed by western blotting.
a) BaF3 and BaF3-p210 cells cultured with and without IL-3 for 24h.
b) TonB201.1 cells cultured in absence of IL-3 for 48h and treated with doxycycline (1μg/ml) to induce BCR/ABL expression, at 0h, 3h, 10h, 24h and 48h prior harvesting.
The lysates (50μg) were fractionated on a 10% SDS-PAGE gel and immunoblotted with the antibodies indicated above.

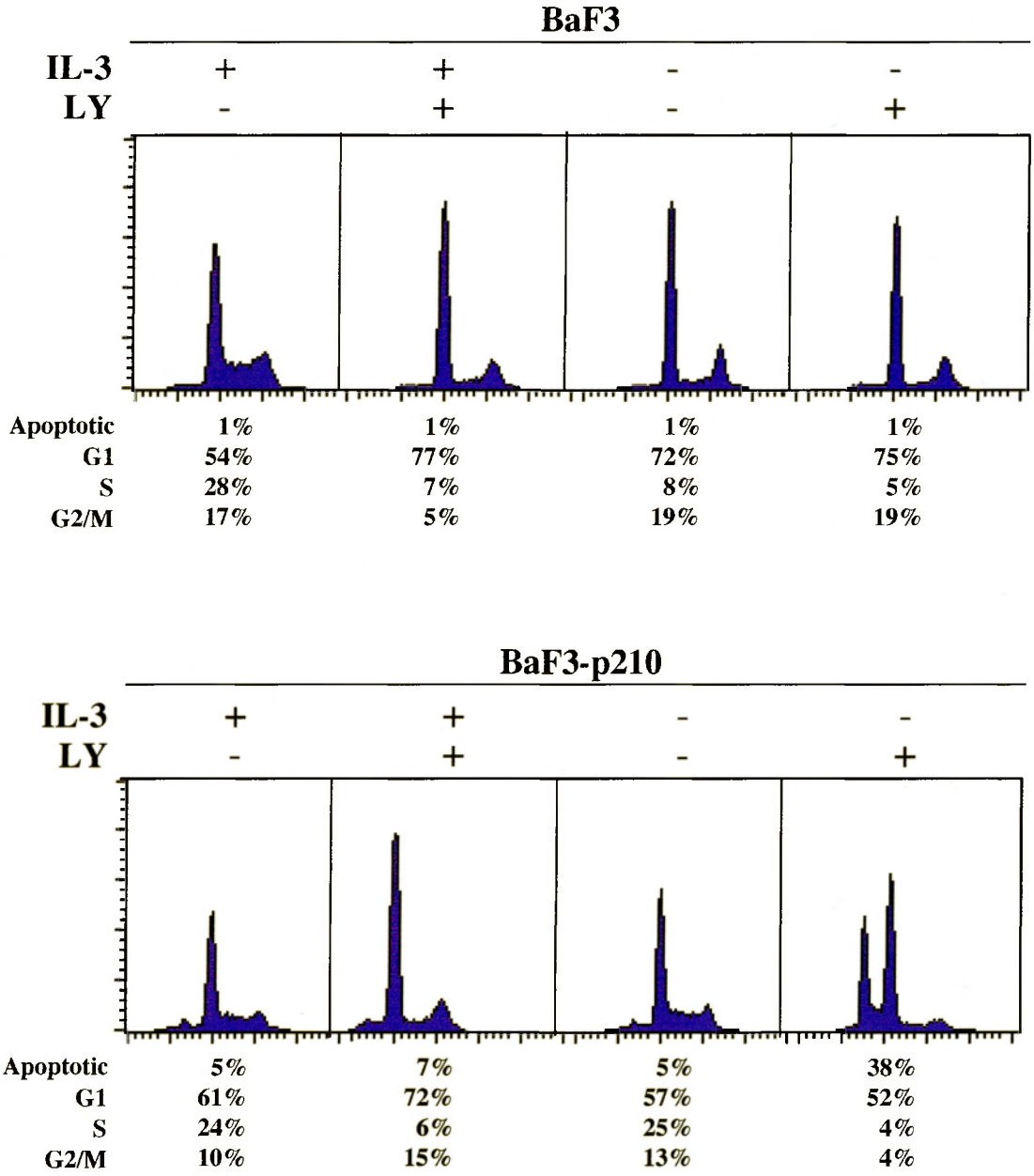

Figure 3.16. PI 3-K inhibitor LY294002 induces cell cycle arrest in BaF3 and BaF3-p210 cells.

Cells were incubated for 24h with LY294002 (50μM) in the presence or absence of IL-3. Cells were harvested and the cell cycle progression was assessed by propidium iodide staining as described in chapter 2.

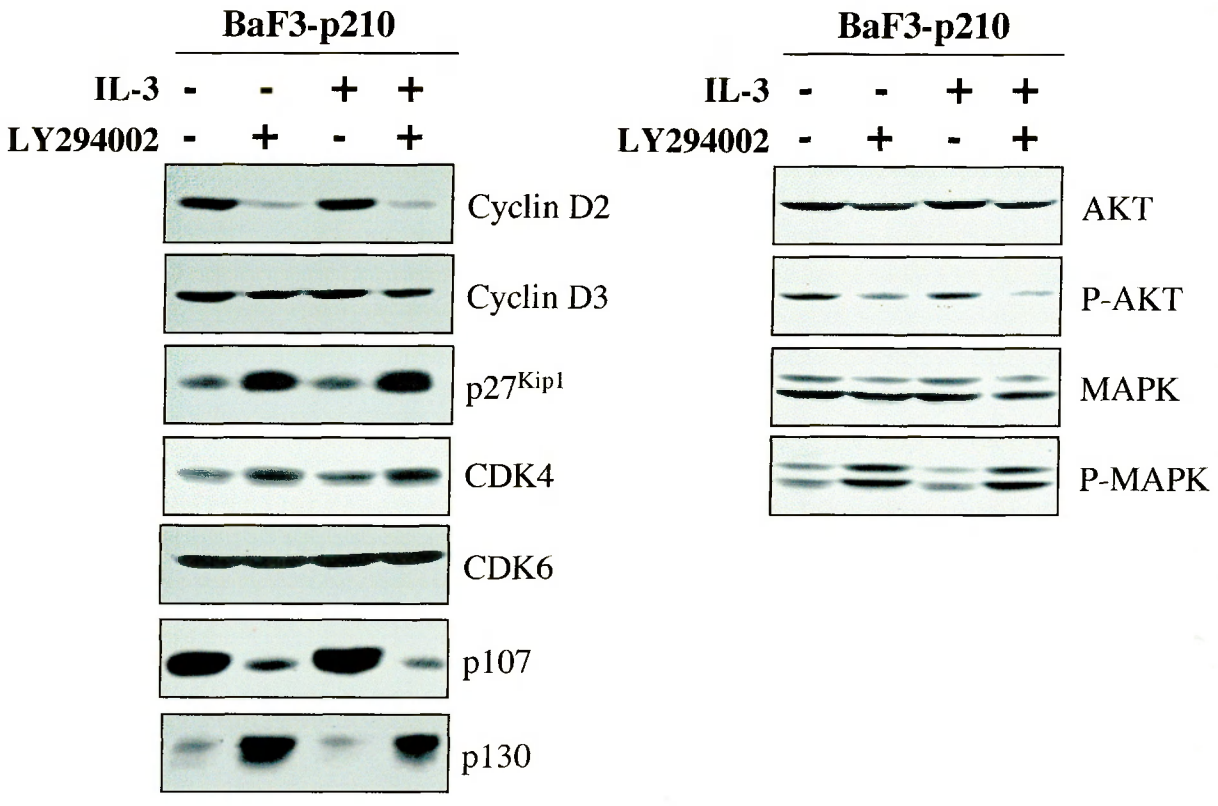

Figure 3.17. Effects of LY294002 on the expression of cell cycle regulators in BaF3-p210 cells

Cells were incubated for 24h with LY294002 in the presence or absence of IL-3. 50μg of total cell extracts were separated by SDS-PAGE, blotted and probed with the antibodies indicated above.

In contrast, phosphorylation of MAPKinase was induced by LY294002, suggesting that PI 3-Kinase has a role in transducing the growth and survival signals derived from IL-3 and BCR/ABL.

The proliferation arrest induced by LY294002 in BaF3-p210 cells could be abrogated by overexpression of cyclin D2. Indeed, after treatment with LY294002 for 24 hours, cyclin D2 stable transfectants were found to proliferate normally with no sign of cell cycle arrest or apoptosis (Figure 3.18). As expected, the control transfectants harbouring the empty expression vector accumulated at the G1 phase of the cell cycle. This result provides further evidence that cyclin D2 is a crucial component of signal transduction pathways downstream from BCR/ABL. Furthermore, these results also imply that cyclin D2 expression is important for cell cycle progression and survival.

Taken together, these results demonstrate that the regulation of cyclin Ds and $p27^{Kip1}$ is mediated, at least partially, *via* the PI 3-Kinase pathway.

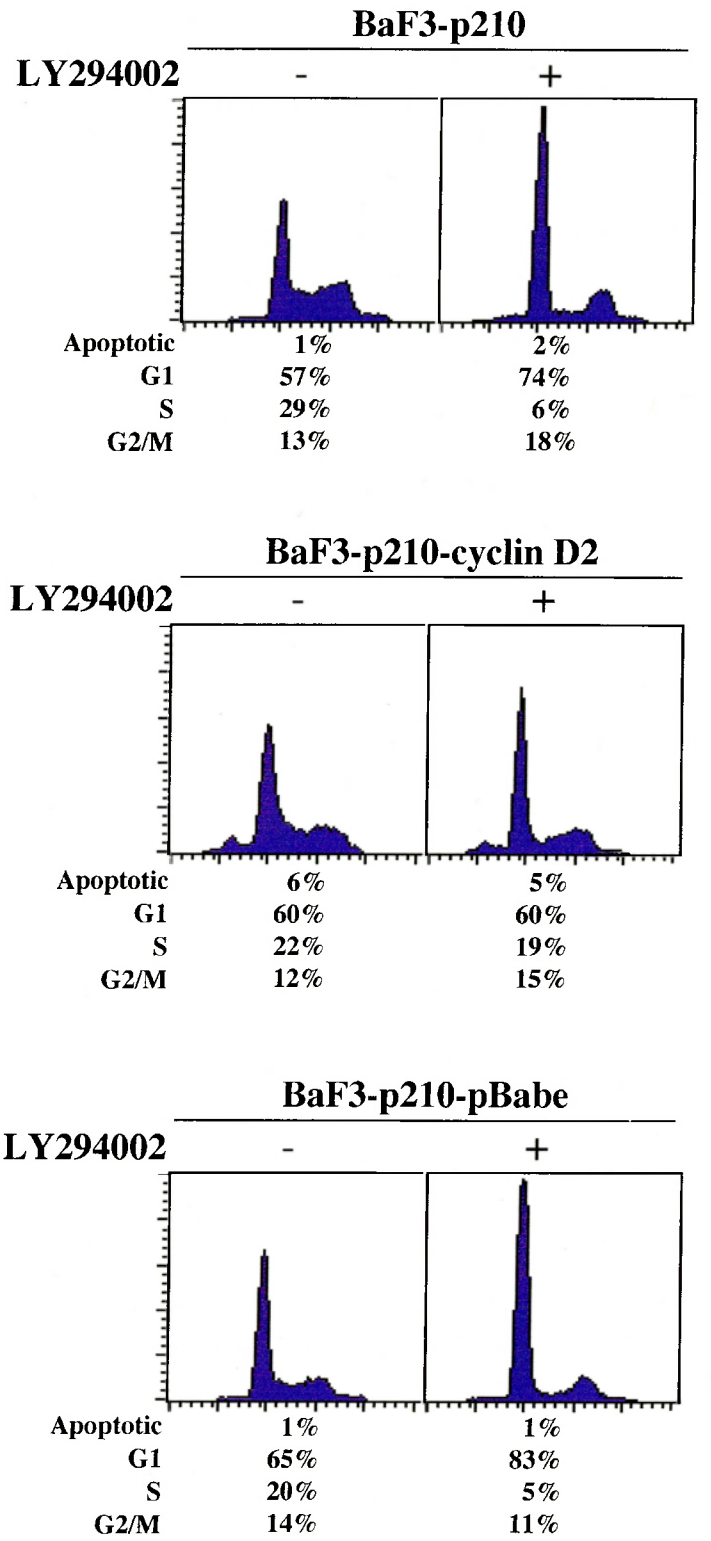

Figure 3.18. Expression of cyclin D2 overcomes the G1 arrest induced by LY294002 in BaF3-p210 cells.

Cells were incubated for 24h with LY294002 (50μM) in the absence of IL-3 and analysed by FACS as described in chapter 2.

Chapter 4. DISCUSSION

The passage of eukaryotic cells through the cell cycle is a highly regulated process involving ordered expression of a series of cell cycle control genes (Paggi *et al.*, 1996; Weinberg, 1995). In haematopoietic cells growth factors, such as interleukin 3 (IL-3), are required to induce commitment of responsive cells to enter the S phase but are not again necessary until the next G1 phase (Reed, 1997). Deprivation of growth factors causes arrest in G1 (Reed, 1997), often followed by apoptosis (Dexter and Heyworth, 1994; Williams *et al.*, 1990). Although much is known about the growth factors of myeloid cells, their receptors and the initial events of signalling, the exact mechanisms and downstream targets which mediate the mitogenic and anti-apoptotic functions of cytokines remain unclear.

Numerous studies have shown that altered expression of cell cycle regulatory proteins through aberrant activation of intracellular signalling pathways is involved in the pathogenesis of various types of malignancies. BCR/ABL has been shown to be one of a select group of oncogenes that is capable of both inhibiting apoptosis and deregulating cell proliferation (Daley and Baltimore, 1988; Daley *et al.*, 1990). It is believed that BCR/ABL activates similar intracellular signalling pathways to IL-3 to promote proliferation and survival in cytokine-dependent haematopoietic cells. Expression of BCR/ABL activates multiple signalling cascades (Cortez *et al.*, 1995), including the Ras (Skorski *et al.*, 1994), phosphatidylinositol 3-kinase (Skorski *et al.*, 1997; Skorski *et al.*, 1995a) and JAK/STAT pathways (Gesbert and Griffin, 2000), which have also been implicated in IL-3-dependent signalling (Coffer *et al.*, 1998a; Coffer *et al.*, 1998b; Reddy *et al.*, 2000). To elucidate the molecular mechanisms and downstream effectors involved in normal and oncogene-induced growth and

survival, the expression levels of molecules along the pRB pathway were studied in the IL-3-dependent pre-B cell line, BaF3, and in a BaF3 cell line stably expressing BCR/ABL, BaF3-p210.

Both IL-3 withdrawal and inhibition of BCR/ABL activity by STI571 treatment in BaF3 and BaF3-p210 cells, respectively, resulted in G1 arrest. This G1 arrest was associated with the appearance of dephosphorylated pRB, indicating an arrest at the G1 restriction point. In agreement with this notion was the finding that the cyclin D- and E-dependent kinase activities were reduced after IL-3 withdrawal or BCR/ABL inhibition. In order to address the mechanism by which the reduction in kinase activity of these complexes occurs, the expression of the cyclins, CDKs and CKIs was analysed. The results demonstrated that IL-3 and BCR/ABL promote haematopoietic cell proliferation and survival through modulation of cyclin D2 and $p27^{Kip1}$ expression. Also, they suggest that this regulation occurs through the PI3-Kinase pathway.

The expression of cyclin D2 and D3 is induced by IL-3 and BCR/ABL

In haematopoietic cells, the D cyclins are particularly attractive candidates for growth regulatory proteins, since a number of studies have emphasised the significance of D cyclins in growth regulation of these cells. Deregulated expression of cyclin D1 has been shown to be oncogenic for lymphoid cells (Rosenberg *et al.*, 1993; Withers *et al.*, 1991) and in primary human T lymphocytes the expression of cyclin D2 and D3 is IL-2 dependent and inducible in G1 by various mitogens (Ajchenbaum *et al.*, 1993). The results obtained in this study suggest that the cyclin Ds are downstream targets of both BCR/ABL and IL-3. Specifically, it was shown that IL-3 withdrawal induced the down-regulation of cyclin D2 and D3, in BaF3 cells. In contrast, in BCR/ABL

expressing cells the cyclin Ds were expressed at high level independently of IL-3 presence. To ensure that the properties observed in the BaF3 cells stably expressing BCR/ABL are related to BCR/ABL expression and not a result of mutations introduced during the establishment of the BaF3-p210 cell line, the findings were corroborated by two different approaches. First, a BaF3 cell line with tetracycline-dependent BCR/ABL expression (TonB210.1) was used, and second, BCR/ABL activity in BaF3-p210 cells was inhibited using the drug STI571. Results from both systems confirmed that the expression of cyclin Ds is associated with BCR/ABL expression in BaF3 cells.

At the molecular level, accumulation of cyclin Ds and down-regulation of $p27^{Kip1}$ in response to IL-3 and/or BCR/ABL expression is associated with the activation of CDK4/CDK6 and CDK2 activity and the phosphorylation of the pocket proteins. The Western blot results confirm the induction of cyclin D and E-dependent kinase activity, represented by hyperphosphorylated pRB (see Figures 3.2, 3.5 and 3.7), in response to IL-3 and/or BCR/ABL, indicating a release of sequestered transcription factors of the E2F family and entry into S phase. Since cyclin D2 is the first G1 cyclin detected in proliferating BaF3 cells, the finding that its expression level parallels the accumulation of the *in vivo* CDK4/6 activity and pocket protein hyperphosphorylation strongly suggested that cyclin D2-CDK4/6 mediates the initial and continuous hyperphosphorylation of pRB during G1. In addition, since CDK4 and 6 are constitutively expressed in B-lymphocytes, these results also indicate that cyclin D2 protein expression could be rate-limiting for cyclin D-CDK4/6 activity and, therefore, the initiation of pocket protein phosphorylation.

It is interesting to notice that although both IL-3 and BCR/ABL appear to target the expression of cyclin D2 and $p27^{Kip1}$ to regulate cell growth and survival, there are considerable differences in the expression levels of certain proteins along the

pRB pathway (Figure 3.2). The primary consequence of cyclin D2 down-regulation is the reduction in CDK4/6 kinase activity. This can be followed by a redistribution of $p27^{Kip1}$ from cyclin D2/CDK4/6 complexes to cyclin E/CDK2, also inhibiting CDK2 activity. In BCR/ABL expressing cells, a pronounced reduction of cyclin D2-associated kinase activity (CDK4/6), compared to the parental cell line levels, was observed, suggesting a down-regulation in cyclin D2 protein levels. However, the levels of cyclin D2 were equivalent in both cell lines, indicating that BCR/ABL fully restored the expression of cyclin D2. A possible explanation for this discrepancy could be that the role of cyclin D2 in these cells is not only CDK activation but also the binding of CDK inhibitors, such as $p27^{Kip1}$. It has been proposed that cyclin Ds can have a role as competitors for $p27^{Kip1}$ binding (Bouchard *et al.*, 1999; Perez-Roger *et al.*, 1999). In response to activation of myc, levels of both cyclin D2 and cyclin D2/CDK4-$p27^{Kip1}$ complexes strongly increase, which accounts for a dissociation of $p27^{Kip1}$ from CDK2. However, in the cells expressing BCR/ABL, $p27^{Kip1}$ levels were almost undetectable, whilst still present at a basal level in the parental cell line in the presence of IL-3.

Hence, cyclin D2 could be fulfilling other roles, rather than sequestering $p27^{Kip1}$, in the BCR/ABL expressing cells. The first hypothesis is that cyclin D2 accelerates $p27^{Kip1}$ degradation by dissociating $p27^{Kip1}$ from cyclin E/CDK2 complexes. Phosphorylation of $p27^{Kip1}$ by CDK2/Cyclin E has been widely observed (Muller *et al.*, 1997; Vlach *et al.*, 1997) and this phosphorylation results in subsequent degradation by the proteasome pathway. Cyclin D2 competing with cyclin E/CDK2 complexes for $p27^{Kip1}$ binding, after $p27^{Kip1}$ phosphorylation by cyclin E/CDK2, could increase $p27^{Kip1}$ turnover by hastening dissociation of phosphorylated $p27^{Kip1}$ from cyclin E/CDK2 complexes, and thus, releasing it for degradation by the ubiquitin/proteasome pathway.

The other hypothesis involves cyclin D2 targeting degradation of $p27^{Kip1}$ by phosphorylation. Other $p27^{Kip1}$ kinases (different from CDK2/cyclin E) can phosphorylate $p27^{Kip1}$ protein and regulate its inhibiting activity and/or its stability. For example, the Kaposis's sarcoma-associated human herpesvirus 8 (KSHV/HHV8) encodes a protein closely related to cellular D-type cyclins. Complexes formed between the viral cyclin and CDK6 can phosphorylate $p27^{Kip1}$ and trigger its degradation (Ellis *et al.*, 1999). In BCR/ABL expressing cells cyclin D2 could play a more active role directly phosphorylating $p27^{Kip1}$, leading to its ubiquitination and subsequent degradation by the proteasome.

Nevertheless, both of the hypotheses described above seem unlikely since there is no evidence documenting these alternative roles for cyclin D2. Therefore the lower levels of phosphorylated pRB in the BCR/ABL expressing cells cannot be attributed to a decrease in cyclin D2 associated activity, and thus another mechanism must be responsible. It is probable that the lower levels of substrate result in lower levels of phosphorylated pRB. In effect, the levels of total pRB are down-regulated in BCR/ABL expressing cells (Figure 3.2), even in the presence of IL-3.This suggests first, that pRB levels are negatively regulated by BCR/ABL, and second, that BCR/ABL mimics IL-3 only partially and consequently other signalling pathways are involved.

The essential role of cyclin D2 for cell cycle progression is documented by the fact that overexpression of cyclin D2 in BaF3 cells prevented the cell cycle arrest as well as the associated apoptosis caused by IL-3 withdrawal. Similarly, BaF3-p210 cells exposed to STI571 can be rescued for cell cycle progression by overexpression of cyclin D2. Using both PI and Annexin V staining, it was demonstrated that ectopic expression of cyclin D2 protects the BaF3-p210 cells from undergoing the G1 cell cycle arrest and subsequent cell death induced by STI571 treatment. This finding implies that cyclin D2 has a role in regulating cell

cycle progression and in cell survival in these haematopoietic cells and that its down-regulation is crucial to the growth arrest and apoptosis. This idea is supported by a recent report showing that the forced expression of another D-type cyclin, cyclin D3, can overcome the proliferation arrest and apoptosis induced by activation of T-cell receptors in leukemic T cells (Boonen *et al.*, 1999).

Since the regulation of growth and differentiation of haematopoietic cells by cytokines involves a careful balance of proliferation and cell death, BCR/ABL targeting cyclin D2 and inducing its anti-apoptotic effects could disrupt the ability of IL-3 to control cell proliferation and viability. In normal haematopoietic cells, the level of cyclin D2 expression and activity is directly dependent on the level of IL-3. As BCR/ABL activity is constitutive, the effect is analogous to constant activation by IL-3. This could explain why leukemic cells expressing BCR/ABL have a growth and survival advantage.

$p27^{Kip1}$ expression is regulated by BCR/ABL and IL-3

Deregulation of cell cycle checkpoints is an almost universal abnormality in human cancers and is most often due to loss-of-function mutations of tumour suppressor genes such as Rb, p53 or p16. In this study I demonstrated that BCR/ABL inhibits the expression of a key cell cycle inhibitor, $p27^{Kip1}$. $p27^{Kip1}$ is a member of a family of cell cycle regulatory proteins that include $p21^{Cip1}$ and $p57^{Kip2}$. $p27^{Kip1}$ is a widely expressed inhibitor of the essential cell cycle kinase CDK2, which regulates entry into S phase (Hsieh *et al.*, 2000). High levels of $p27^{Kip1}$ inhibit the activity of the CDK2-cyclin E complex and prevent phosphorylation of critical target molecules necessary for initiation of S phase, including Rb. Phosphorylation of Rb is necessary for release of sequestered transcription factors of the E2F family and induction of E2F-dependent gene

expression (Dyson, 1998). In normal cells, progression through G1/S phase requires $p27^{Kip1}$ to be displaced from CDK2, either by sequestration in cyclin D-CDK4 complexes, which are not inhibited by $p27^{Kip1}$ (Reynisdottir *et al.*, 1995), or by down-regulation of the protein through multiple mechanisms. Extensive studies during the past years provided compelling evidence that $p27^{Kip1}$ has a critical role in carcinogenesis, since $p27^{Kip1}$-deficient and hemizygote mice develop spontaneous tumours (Fero *et al.*, 1996). Moreover, in human tumours the levels of $p27^{Kip1}$ protein strongly correlate with prognosis (Esposito *et al.*, 1997).

In the studies reported here, expression of BCR/ABL was shown to specifically and rapidly decrease expression of $p27^{Kip1}$, coincident with progression from G1 into S phase. The down-regulation of $p27^{Kip1}$ was shown to be consequent on BCR/ABL activity using two different approaches, that were also used to investigate cyclin D2 regulation, above. First, induction of BCR/ABL by a tetracycline-regulated promoter was associated with a down-regulation of $p27^{Kip1}$. Second, inhibition of BCR/ABL kinase activity with the ABL tyrosine kinase inhibitor STI571 specifically increased $p27^{Kip1}$ levels.

To examine the functional significance of $p27^{Kip1}$ up-regulation in BaF3 cells, biologically active $p27^{Kip1}$ was transduced into BaF3 cells using a TAT-fusion protein. The results showed that forced expression of $p27^{Kip1}$ is sufficient to induce cell cycle arrest and apoptosis in BaF3 cells. Moreover, acute overexpression of $p27^{Kip1}$ inhibited the proliferation of BCR/ABL positive cells, indicating that BCR/ABL induced transformation is dependent on the level of $p27^{Kip1}$ protein. It is notable that only the wild type, but not a mutant $p27^{Kip1}$, which cannot interact with CDK2, can promote cell cycle arrest and apoptosis in BaF3 and BaF3-p210 cells, indicating that the ability of $p27^{Kip1}$ to bind and inhibit CDK2 is important for mediating cell cycle arrest and apoptosis.These results support the notion that

$p27^{Kip1}$ might function as a tumour suppressor gene not only because it works as a negative regulator of cell cycle progression, but also because it is associated with the induction of apoptosis.

Molecular mechanisms of cyclin D2, D3 and $p27^{Kip1}$ regulation of expression

Northern blot analysis revealed that the expression of both cyclin D2 and $p27^{Kip1}$ in response to IL-3 and/or BCR/ABL are, at least partially, regulated at the transcriptional level. In contrast, the expression of cyclin D3 appeared to be regulated predominantly at a post-transcriptional level.

Although cyclin D3 protein levels were induced by BCR/ABL and IL-3, the levels of cyclin D3 mRNA seemed to be independent of IL-3 stimulation and were strongly down-regulated upon BCR/ABL expression. This paradox suggests that both IL-3 and BCR/ABL regulate the expression of cyclin D3 at the post-transcriptional level, but the regulation is achieved using different mechanisms. A rapid increase in the translation of cyclin D3 may be essential for the positive regulation of the protein expression in response to either IL-3 or BCR/ABL. Alternatively, regulation of the half-life of the protein, by protein stabilisation or decreased degradation, could be important for the accumulation of cyclin D3 in the absence of increased transcription. However, further studies will be needed to prove this notion conclusively.

The mechanism of down-regulation of $p27^{Kip1}$ is likely to be of interest. $p27^{Kip1}$ protein expression level is known to be regulated by several mechanisms, including degradation by the ubiquitin/proteasome pathway (Esposito *et al.*, 1997), phosphorylation (Vlach *et al.*, 1997) and subcellular compartmentalisation (Orend *et al.*, 1998).

Although most studies have shown that $p27^{Kip1}$ expression is mainly regulated at the post-transcriptional level by controlling degradation of this protein (Hengst and Reed, 1996; Vlach *et al.*, 1997), a small number of studies have implied that $p27^{Kip1}$ might also be regulated at the transcriptional level (Dijkers *et al.*, 2000; Medema *et al.*, 2000). Interestingly, the latter study has proposed a mechanism involving PI 3-Kinase-mediated inactivation of Forkhead transcription factors by phosphorylation. The Forkhead transcription factors AFX, FKHR, and FKHR-L1 are orthologues of DAF-16 of *Caenorhabditis elegans* and have previously been shown to be involved in regulating viability and G1 to S progression (Kenyon *et al.*, 1993; Ogg *et al.*, 1997). AFX and FKHR-L1 have been shown to up-regulate $p27^{Kip1}$ promoter activity (Dijkers *et al.*, 2000; Medema *et al.*, 2000). The Forkhead factors are exported from the nucleus in response to an AKT-dependent phosphorylation, thus suggesting the model that transcription of $p27^{Kip1}$ is decreased when AKT, or a kinase regulated by AKT, phosphorylates one or more Forkhead transcription factors.

In contrast, recent work has shown that BCR/ABL promotes degradation of $p27^{Kip1}$ through a proteasome-dependent degradation pathway (Gesbert *et al.*, 2000; Jonuleit *et al.*, 2000). Proteasome-dependent degradation of other proteins has also been shown to be important for BCR/ABL transformation (Dai *et al.*, 1998). During progression through the cell cycle, $p27^{Kip1}$ is known to be phosphorylated by cyclin E/CDK2 complexes (Vlach *et al.*, 1997) on Thr-187, which targets this CDK inhibitor for degradation via the ubiquitin/proteasome pathway. Lactacystin, a cell permeable proteasome inhibitor, completely prevented the BCR/ABL-mediated $p27^{Kip1}$ down-regulation in BaF3 cells, implying that BCR/ABL interferes with an ubiquitin regulated proteasomal degradation pathway of the $p27^{Kip1}$ protein (Jonuleit *et al.*, 2000). Similar results were obtained using a different proteasome inhibitor, *N*-acetyl-leucyl-leucine

norleucinal (Gesbert *et al.*, 2000). These data are consistent with previous findings showing that cyclin D/CDK4/6 activity can activate cyclin E expression *via* E2F (Geng *et al.*, 1999; Hurford *et al.*, 1997; Lukas *et al.*, 1997) and that phosphorylation of $p27^{Kip1}$ by the induced cyclin E/CDK2 results in its degradation via the ubiquitin/proteasome pathway (Vlach *et al.*, 1997). It is therefore conceivable that expression of cyclin D2 induces down-regulation of $p27^{Kip1}$ through stimulation of cyclin E synthesis rates and thus cyclin E-CDK2 activity.

Although a down-regulation of $p27^{Kip1}$ mRNA levels was observed in cells expressing BCR/ABL, this down-regulation was not as pronounced as the down-regulation at the protein levels and cannot account for the dramatic reduction of protein. In other cell systems, $p27^{Kip1}$ regulation has been shown to occur at both transcriptional and post-trancriptional levels (Servant *et al.*, 2000). $p27^{Kip1}$ down-regulation by BCR/ABL may therefore involve both down-regulation of its mRNA and an enhanced degradation of the protein by the ubiquitin/proteasome pathway.

Cyclin D2 and $p27^{Kip1}$ expression are regulated through a PI 3-K-dependent pathway

Although BCR/ABL is known to have prominent growth promoting effects, the pathways involved in its ability to activate mitogenic signalling are unclear. Having established cyclin D2 and $p27^{Kip1}$ as important targets of BCR/ABL and IL-3, I next tried to identify the pathways that are activated leading to cyclin D2 and $p27^{Kip1}$ regulation. Various signal transduction pathways have been shown to be activated by IL-3 and BCR/ABL, amongst them the Ras/Mitogen-activated protein Kinase (MAPKinase), JAK/STAT and the PI 3-Kinase pathways.

A potential role for PI 3-K in mediating the proliferative response (targeting cyclin D2 and $p27^{Kip1}$) from IL-3 and BCR/ABL was suggested by the observation that the expression of BCR/ABL resulted in an increase of phosphorylation of the serine/threonine kinase AKT. AKT is thought to have a role in proliferative and anti-apoptotic cell responses by regulating the activity of several proapoptotic proteins (Marte and Downward, 1997), for example BAD (Datta *et al.*, 1997), the protease caspase 9 (Cardone *et al.*, 1998) and FKHR-L1 (Brunet *et al.*, 1999). AKT is activated by IL-3 in a PI 3-Kinase-dependent manner (del Peso *et al.*, 1997; Franke *et al.*, 1995). To explore this idea, the effect of the PI 3-K inhibitor LY294002 in BaF3 and BaF3-p210 cells in the presence or absence of IL-3 were analysed. Inhibition of PI 3-Kinase activity was found to induce apoptosis in both BaF3 and BaF3-p210 cells, and this was associated with down-regulation of cyclin D2 levels and up-regulation of $p27^{Kip1}$ expression.

The PI 3-K enzyme is a heterodimer composed of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit containing two SH2 domains (N- and C-terminal) and one SH3 domain (Kapeller and Cantley, 1994). Activation of PI 3-K by many growth factor receptors involves recruitment of the enzyme to the membrane through binding of one or both of the SH2 domains to specific pYXXM (pY, phosphorylated tyrosine) motifs in the receptor or in phosphorylated adapter molecules. There is abundant evidence that PI 3-K is activated in cells expressing BCR/ABL (Jain *et al.*, 1997; Varticovski *et al.*, 1991) and that this activation is important for BCR/ABL transformation. Previously, Skorski et al. (1997) have investigated the role of the PI 3-Kinase in mediating BCR/ABL-induced cell growth and survival by using antisense oligonucleotides and the PI 3-K inhibitor wortmannin. They have reported that BCR/ABL can neither induce cell proliferation nor survival when PI 3-K is inhibited. Furthermore, they showed that one of the targets of PI 3-K, the AKT kinase, was likely to be involved in

BCR/ABL transformation, since dominant negative mutants of AKT also inhibited BCR/ABL-induced transformation *in vitro* and *in vivo*.

The results obtained in this study indicate that cyclin D2 and $p27^{Kip1}$ are downstream targets of PI 3-K, in accordance with other reports recently published: Gesbert et *al.* (2000), propose that BCR/ABL activates AKT through PI 3-K, resulting in a significant down-regulation of $p27^{Kip1}$ and accelerated entry into S phase. $p27^{Kip1}$ expression was suppressed in BaF3 cells by an activated mutant of AKT (AKT fused to a membrane-targeting sequence, HA-AKT-CAAX, whereas overexpression of a non-activated AKT (HA-AKT) had no effect. These results showed that activation of AKT by itself was capable of regulating $p27^{Kip1}$ levels. The same results were obtained in cells exposed to LY-294002 to block PI 3-K activity, indicating that AKT functions downstream of PI 3-K. Simultaneously, another team used cells expressing temperature sensitive BCR/ABL to prove down-regulation of $p27^{Kip1}$ by BCR/ABL via PI 3-Kinase in BaF3 cells and human M07 cells (Jonuleit *et al.*, 2000).

The results presented in this study suggest that BCR/ABL acts as a positive regulator for the PI 3-Kinase/AKT signalling pathway, which controls and co-ordinates two major cellular processes: cell cycle progression and cell death.

Hypothetical model

Overall, the studies reported here describe a new signalling pathway for BCR/ABL and IL-3 to promote cell survival and proliferation. The regulation of cyclin D2 and $p27^{Kip1}$ through PI 3-K and AKT is likely to be a significant component of this activity.

A simple model of this signalling pathway is presented below, based on the integration of the results obtained in this study and published research. However,

as mentioned previously, IL-3 and BCR/ABL do not induce identical responses, therefore alternative signalling pathways might be used by either molecule to promote cell survival and proliferation.

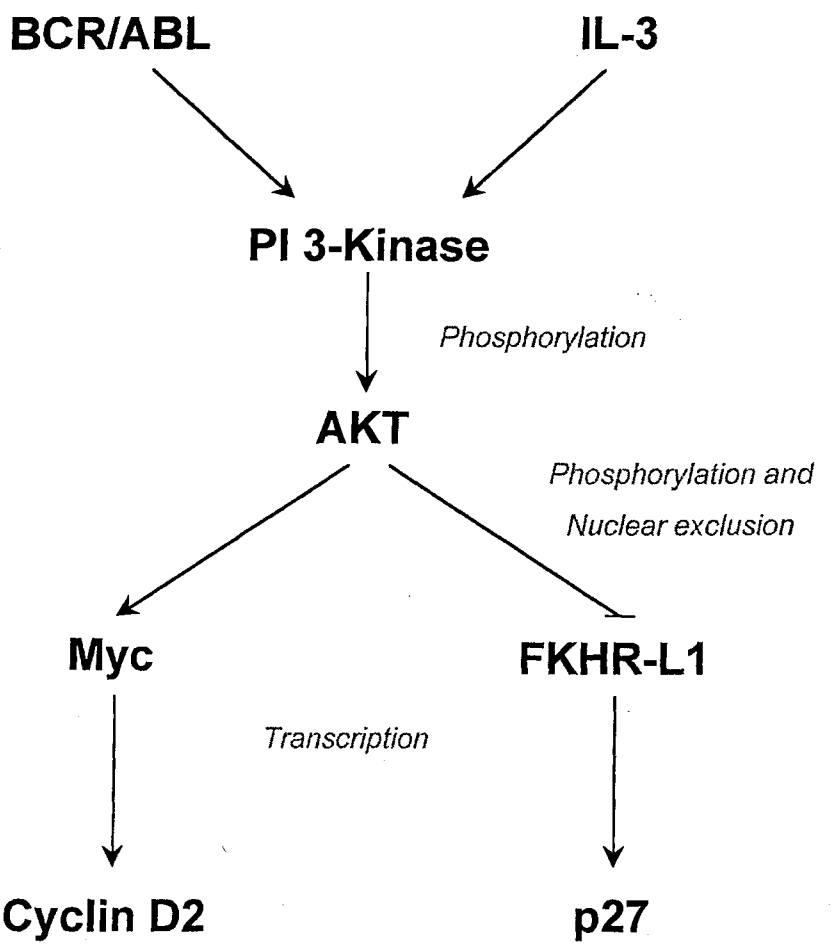

Figure 4.1. A model of BCR/ABL and IL-3 signalling.

Although PI 3-K is known to be activated by v-abl and BCR/ABL (Varticovski *et al.*, 1991), the mechanism remains unclear since abrogation of the interaction between BCR/ABL and p85 PI 3-K does not impair PI 3-Kinase activation or BCR/ABL induced growth factor independence of haematopoietic cells (Jain *et al.*, 1996). Using cells expressing mutant, temperature-sensitive p210 BCR/ABL

in which the tyrosine in the YXXM motif of p210 BCR/abl was replaced by histidine it was demonstrated that abolition of the binding of BCR/ABL to p85 SH2 was not required for BCR/ABL-induced activation of PI 3-Kinase. Despite the lack of direct interaction with p85 SH2 domains, expression of ts p210 BCR/abl resulted in rapid, time-dependent activation of total and membrane-associated PI 3-kinase and increased PI 3-kinase activity in anti-P-tyr and anti-abl immunoprecipitates. Therefore, regulation of PI 3-Kinase activity by BCR/ABL might involve interaction with other tyrosine phosphorylated intermediate proteins. Significantly, activation of the kinase activity in BCR/ABL leads to phosphorylation of several intracellular substrates, including Shc, CRKL and p120cbl.

CRKL is a 39kDa SH2, SH3 domain-containing adapter protein related to the *CRK* oncogene of the avian sarcoma virus, CT10. c-*CBL* (Casitas B-lineage Lymphoma) is the cellular homolog of v-*CBL*, the transforming protein of the CAS NS-1 retrovirus which can directly cause myeloid and lymphoid leukemias in mice (Langdon *et al.*, 1989). p210cbl is involved in signal transduction pathways in normal cells associated with proliferation or activation. For example, p210cbl is a common substrate of tyrosine kinases activated after haematopoietic cytokine receptor stimulation (Sattler *et al.*, 1996).

It has been proposed that a multimeric complex of signalling proteins including cbl, crkl or crk, and grb2 links BCR/ABL to the PI 3-Kinase pathway (Figure 4.2). In BCR/ABL transformed BaF3 cells, cbl is a major tyrosine phosphorylated protein, and tyrosine phosphorylation of cbl leads to an increase in association with grb2 and p85 PI 3-K. Moreover, this association resulted in enhanced PI 3-Kinase activity (Jain *et al.*, 1997). Immunoprecipitates from BCR/ABL transformed cell lines contained the p85 subunit of PI 3-Kinase and the adapter proteins CRKL and CRK. *In vitro* binding studies indicated that the SH2 domains

of CRKL bind directly to cbl, while the SH3 domains bind to BCR/ABL (Sattler *et al.*, 1996). Altogether, these results permit to establish a model for the activation of PI 3-K by BCR/ABL.

Downstream proteins recruited by PI 3-K upon stimulation by BCR/ABL include AKT. The involvement of PI 3-K in regulating AKT was initially suspected following observations that the activation of AKT induced by growth factors was inhibited by wortmannin, a chemical inhibitor of PI 3-Kinase (Franke *et al.*, 1995), and also by the expression of a dominant-negative form of PI 3-Kinase regulatory subunit, Δp85 (Burgering and Coffer, 1995). The regulation of AKT by PI 3-Kinase was confirmed afterwards: synthesis of constitutively activated forms of PI 3-Kinase resulted in stimulation of AKT (Franke *et al.*, 1995; Klippel *et al.*, 1996). The essential role of AKT in mediating BCR/ABL leukemogenesis was established both *in vitro* and *in vivo* by experiments demonstrating that a dominant-negative Akt mutant inhibited BCR/ABL induced transformation of bone marrow cells and suppressed leukemia development in mice (Skorski *et al.*, 1997).

Similarly, IL-3 has been shown to activate the AKT kinase through a PI 3-Kinase-dependent pathway (Coffer *et al.*, 1998b). Stimulation with IL-3 increased AKT activity and was inhibited by wortmannin and LY294002, two chemical inhibitors of PI 3-K activity, in the lymphoid progenitor cell line FL5.12 (del Peso *et al.*, 1997). Dominant-negative mutants of AKT specifically block AKT activation by IL-3 and interfere with IL-3-dependent proliferation. Moreover, overexpression of AKT or oncogenic v-akt protects 32D cells from apoptosis induced by IL-3 withdrawal (Songyang *et al.*, 1997).

c-*myc* has been identified as a potential target of AKT and PI 3-K (Skorski *et al.*, 1997). Interestingly, overexpression of myc in BaF3 cells is sufficient to induce progression through the cell cycle (Ahmed *et al.*, 1997) and BCR/ABL

transformation seems to require overexpression of c-*myc*, since a dominant-negative c-Myc blocks transformation by BCR/ABL (Sawyers *et al.*, 1992). Moreover, BCR/ABL has been shown to upregulate c-Myc at the transcriptional level (Cortez *et al.*, 1995; Pendergast *et al.*, 1993; Stewart *et al.*, 1995).

The proto-oncogene c-*myc* is a key regulator of cell cycle proliferation and apoptosis. Myc is rapidly induced by growth factors and prevents exit from the cell cycle. c-*myc* has long been implicated in the pathogenesis of B-lineage neoplasia, notably Burkitt lymphoma in humans, plasmacytoma in the mouse and immunocytoma in the rat (Klein and Klein, 1986; Magrath, 1990). c-*myc* encodes a basic helix-loop-helix leucine zipper transcription factor that dimerises with Max and binds to specific sequences on DNA, called E-boxes (Henriksson and Luscher, 1996). Myc represents a potential candidate to mediate the signal from AKT to cyclin D2, since the expression of cyclin D2 has been shown to be directly regulated by Myc. The cyclin D2 promoter is repressed by Mad-Max complexes and de-repressed by Myc via a single highly conserved E-box element (Jun *et al.*, 1997).

The induction of cyclin D2 by Myc has been shown to contribute to cell cycle progression and sequestration of $p27^{Kip1}$ (Bouchard *et al.*, 1999; Perez-Roger *et al.*, 1999). In response to activation of Myc, levels of cyclin D2 and cyclin D2-$p27^{Kip1}$ complexes strongly increased. Furthermore, Myc has been shown to abrogate the growth arrest induced by $p27^{Kip1}$ by preventing association of $p27^{Kip1}$ with cyclin E/CDK2 (Vlach *et al.*, 1996).

After induction by Myc, cyclin D2 could directly stimulate cell progression by phosphorylation of pRB. Also, cyclin D2 could inhibit $p27^{Kip1}$ activity by either dissociation of $p27^{Kip1}$ from CDK2/cyclin E complexes, or induction of cyclin E expression and consequent phosphorylation and degradation of $p27^{Kip1}$ by the ubiquitin/proteasome pathway (Muller *et al.*, 1997).

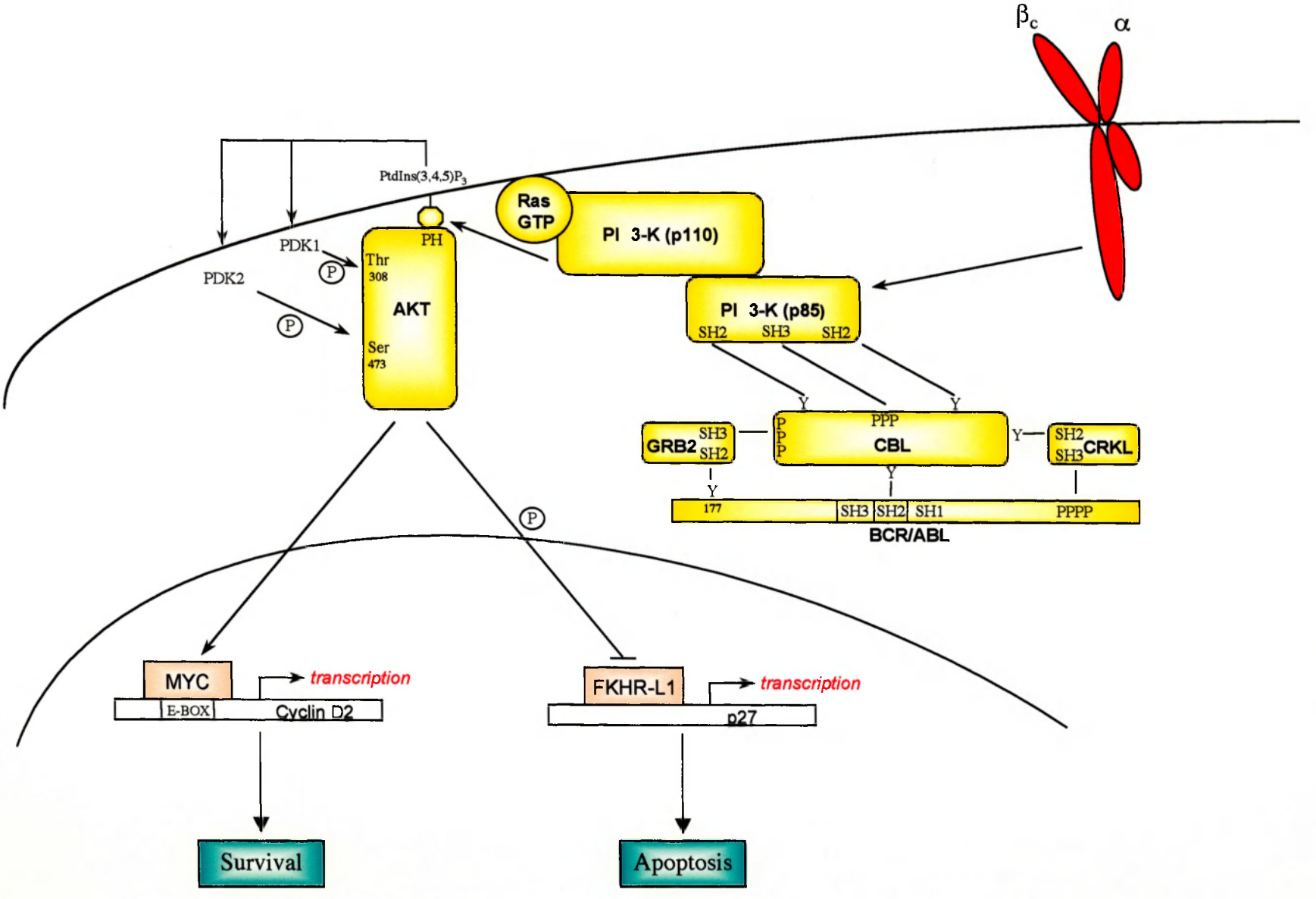

Figure 4.2. Schematic representation of signal transduction pathways activated by IL-3 and BCR/ABL

In parallel, the regulation of $p27^{Kip1}$ could occur at the transcriptional level. The Forkhead factors FKHR-L1 and AFX, are targets of signalling by AKT (Brunet *et al.*, 1999; del Peso *et al.*, 1999; Kops and Burgering, 2000) and have been shown to modulate cytokine-dependent transcriptional regulation of $p27^{Kip1}$ (Figure 4.2) (Dijkers *et al.*, 2000; Medema *et al.*, 2000). IL-3 stimulation in BaF3 cells results in a rapid transient phosphorylation of endogenous FKHR-L1, that can be abrogated by inhibition of PI 3-kinase by LY294002. As a result of its phosphorylation, FKHR-L1 is translocated from the nucleus to the cytoplasm and its transcriptional activity is abolished.

Similar models of action have been proposed for cytokines in different systems. For example, the PI 3-K/AKT pathway has been proposed to be crucial for proliferation in IL-2 signalling system. Ahmed *et al.* (1997) demonstrated that IL-2 stimulates progression through the cell cycle and inhibits apoptosis in BaF3 cells through activation of AKT, which in turn induces the expression of c-myc. More recently, the IFN-α has been shown to induce rapid phosphorylation of both AKT and its substrate FKHR to promote cell survival in primary B-lymphocytes (Ruuth *et al.*, 2001). In addition, IFN-α stimulation of anti-IgM activated cells resulted in down-regulated expression of $p27^{Kip1}$. Another cytokine that can promote cell survival through PI 3-K-mediated activation of AKT is the Tumor Necrosis Factor (TNF). Pastorino *et al.* (1999) demonstrated that, under no cytotoxic conditions, the TNF acts as other growth factors. They showed that TNF induces phosphorylation of the proapoptotic protein BAD and its subsequent translocation from the mitochondria to the cytosol to promote cell survival through PI 3-Kinase-mediated activation of AKT.

The results suggest that the functional substitution of IL-3 by BCR/ABL is due to the constitutive activation of proteins involved in IL-3 signal transduction, and that PI 3-Kinase is essential for cell survival and proliferation. However, there are differences in the effects induced by IL-3 and BCR/ABL, for example the down-regulation of pRB and $p27^{Kip1}$ protein levels by BCR/ABL, which indicate that there are additional signalling pathways involved, and that they are not identical. As summarised at the beginning of this chapter several other pathways of cellular regulation are activated by IL-3 and BCR/ABL. It is likely that BCR/ABL and IL-3 exert their full growth-promoting activities through the simultaneous activation of multiple downstream signalling pathways and that these pathways interact. The relative importance of the signalling pathway seems to depend on the cellular context, what could explain the number of controversial publications on the role of different pathways in cell survival and proliferation. In several cases, the individual inhibition of each pathway is not sufficient to completely abrogate cytokine mediated cell survival, suggesting that cooperation between the pathways is required.

According with this hypothesis, a recent publication suggests a cooperation between the PI 3-K and STAT5 pathways to promote IL-3 mediated suppression of apoptosis in BaF3 cells. Rosa Santos *et al.* (2000), showed that the individual inhibition of STAT5 or PI 3-Kinase weakly affected the IL-3-dependent survival in BaF3 cells. However, the simultaneous inhibition of STAT5 and PI 3-K activities reduced the IL-3 dependent survival of BaF3. In addition, they found an interaction between STAT5 and the p85 subunit of the PI 3-Kinase after IL-3 stimulation. In summary, they propose that IL-3 induces the coordinated activation of distinct signalling pathways that are involved in cell survival by regulating the proper balance between pro- versus anti-apoptotic molecules . Among the other pathways suggested to be involved in both IL-3 and BCR/ABL

signalling is Ras. Ras is a critical signal mediator as a potent suppressor of apoptosis. It has been shown to be activated in response to IL-3 stimulation in BaF3 cells (Satoh *et al.*, 1991). However, in a later study it was demonstrated that although the Ras-dependent pathway is likely to play an important role to facilitate the proliferation, it is dispensable for IL-3-induced growth stimulation (Terada *et al.*, 1995). Analogously, BCR/ABL has been shown to inhibit apoptosis through activation of a Ras-dependent signalling pathway (Cortez *et al.*, 1996). Ras could induce the activation of PI 3-K since the latter has been proposed to be a direct target of Ras in other systems (Kauffmann-Zeh *et al.*, 1997; Marte *et al.*, 1997).

In summary, it is conceivable that several pathways play important roles to facilitate proliferation although they may not be essential for IL-3 or BCR/ABL-stimulated antiapoptotic signal transduction.

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